Application Notes Binder

IV

Innovative microplate readers for all assay needs

BMG LABTECH

The Microplate Reader Company

World-class innovation.
German engineering.
Local support.

Since its establishment in 1989 in Offenburg, Germany, BMG LABTECH has become a world leader in microplate reading thanks to continuous development of innovative and unique technologies.

For more than 25 years, BMG LABTECH has been ruled by its corporate values. Precision, reliability and robustness define the character of our company and permeate every aspect of our work, from our microplate readers to our renowned customer support.

Besides our over 25 year-long expertise in microplate readers, a further guarantee of our success relies on the development, manufacture and quality control of every reader at our headquarters in Germany.

Yet, a perfectly German-engineered instrument is only one part of the solution. It needs to effectively perform all applications. Therefore, BMG LABTECH continuously collaborates with all of the leading reagent companies to optimize reader settings for their assays.

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The application notes detailed herein were all performed on BMG LABTECH microplate readers and outline a variety of experiments and methods.

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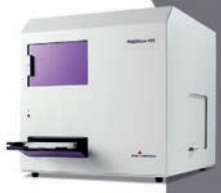
If you have any question regarding assays or techniques, contact your local BMG LABTECH office. Our scientists and engineers are always ready to help!

BMG LABTECH

The Microplate Reader Company

For every application there is a BMG LABTECH reader

High-Throughput Screening



The **PHERAstar® *FSX*** is the ultimate multi-mode reader for high-throughput screening, combining the highest sensitivity with the fastest read times. Thanks to the unique Optic Module system, Simultaneous Dual Emission, UV/Vis spectrometer, and dedicated Alpha Technology and TRF lasers, whatever your application, the PHERAstar *FSX* will perform it with ease and speed, even in 3456-well microplates.

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Assay development

The **CLARIOstar®** is a high-performance monochromator-based multi-mode reader. BMG LABTECH's revolutionary LVF Monochromator™ technology ensures filter-like performance without compromising on flexibility. The CLARIOstar has six detection modes, continuously adjustable bandwidths from 8-100 nm, an Alpha Technology laser, direct optic bottom reading, up to two reagent injectors, a UV/Vis spectrometer. It can handle up to 1536-well microplates.

Life science

The **Omega Series – FLUOstar®, POLARstar®, SPECTROstar®,** and **LUMIstar®** are upgradable multifunction instruments that can support up to six detection modes. Unique features include an ultra-fast UV/Vis spectrometer, Simultaneous Dual Emission detection, injection at the point of measurement and well scanning.

The **SPECTROstar® *Nano*** is an innovative absorbance microplate reader that has the flexibility to perform assays quickly and easily in microplates, cuvettes and small-volume samples. This spectrometer-based reader captures a full UV-visible spectrum in less than 1 sec/well, making it the ideal microplate reader for absorbance measurements.

The **Atmospheric Control Unit (ACU)** module actively regulates both CO_2 and O_2 in the microplate measurement chamber and is available for **CLARIOstar , NEPHELOstar *Plus*,** and the **Omega series** of readers.

For more information on BMG LABTECH's microplate readers visit:
http://www.bmglabtech.com/en/products/

Absorbance

FI + FRET

Table of contents

LUMI + BRET

TRF + TR-FRET

FP

AlphaScreen®

Nephelometry

Glossary

High-throughput determination of bacterial growth kinetics using a BMG LABTECH microplate reader

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- **Growth rates of biocide resistant mutants of salmonella and their parent strains were determined and compared**
- **Growth curves were monitored in absorbance mode automatically over 24 h**
- **Biocide exposure selects for strains with increased tolerance to biocides and there is no obvious fitness cost**

Introduction

Salmonella enterica serovar Typhimurium are amongst the leading causes of gastrointestinal disease. Multiple antibiotic resistance (MAR) in salmonella has been increasing and over 30% of isolates of S. Typhimurium. Efflux is one mechanism that can confer MAR, efflux pumps are membrane proteins that actively export a wide range of toxic substrates including antibiotics, dyes and biocides to the cells external environment, thereby preventing accumulation of toxic agents and mediating resistance to these agents as a result.
The wide spectrum of substrates recognized by efflux systems has prompted concern that exposure of a bacterium to one substrate could select for over-expression of an efflux system and consequent resistance to all other substrates. It is possible that exposure of a bacterium to a biocide could result in selection of an efflux mutant which has reduced susceptibility to antibiotics, which it has not previously encountered. In this study, mutants of salmonella resistant to biocides were selected. The growth rates of biocide resistant mutants were determined and compared to parent strains and the effect of the addition of biocides during growth were determined.

Materials & Methods

Selection of biocide resistant mutants
Previously, a panel of *Salmonella Typhimurium* was used to select biocide resistant mutants. SL1344 was used as a reference control strain, L108 is a derivative of SL1344 lacking the TolC porin and L358 is a multiple antibiotic resistant DT104 isolate. L378 is a ciprofloxacin resistant isolate from poultry and L357 is a representative DT104 isolate. The biocides AQAS (a quaternary ammonium compound), Superkill (a mix of aldehydes), Farm Fluid S (a mixture of tar oils and phenolics) and Virkon S (peroxygen generating compound) were used to select for tolerant mutants by incorporation in agar and overnight incubation after inoculation with each strain.

Analysis of growth kinetics of biocide tolerant mutants
The rate of growth was determined over 24 h at 37°C using a BMG LABTECH microplate reader. 100 µL of sterile LB broth was dispensed into clear sterile 96-well microplates and inoculated with overnight culture of each strain to give a final inoculum of 4%. Readings were taken every ten minutes of absorbance of each well (scanned at 600 nm) in the microplates over the 24 h time period. Each strain was analysed in triplicate wells on at least three separate occasions to give nine data sets for analysis. Additionally, each strain was challenged with either 0.5 X, or the MIC (minimum inhibitory concentration) of the selective biocide for the parent after two hours growth in order to determine whether the inhibitory ability of each biocide was reduced in mutants, respective to parent strains.

Results & Discussion

None of the biocide selected mutants were compromised in their ability to grow. Mutants derived from L358 (S2, S22, S23) after exposure to Superkill grew significantly better than L358 in the absence of biocide (Figure 1).

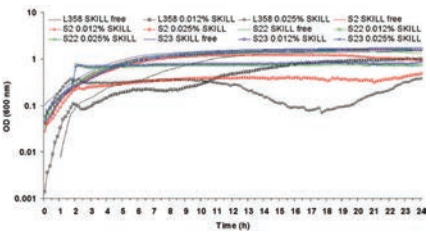

Fig. 1: Growth of Superkill selected mutants S2, S22, S23 and parent L358 at 37°C after inoculation with 4% vol/vol of overnight culture. Superkill was added to cultures at 2 h.

The majority of biocide selected mutants were more resistant to the addition of biocides to the media than their respective parent strains, including those strains for which the MIC of the selective biocide had remained unchanged when compared to the parent (table 1). AQAS selected mutant A27 and A26 were able to grow significantly ($p > 0.01$) better upon both the addition of 0.5 X and the MIC of L378 and L108, respectively, to the media (figures 2 and 3).

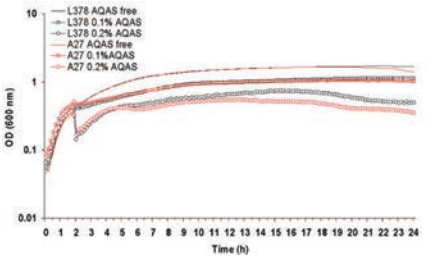

Fig. 2: Growth of AQAS selected mutant A27 and parent L378 at 37°C after inoculation with 4% vol/vol of overnight culture. AQAS was added to cultures at 2 h.

Rev. 05/2006
Keywords: **Bacterial growth, MIC, Salmonella, Superkill, Virkon S**

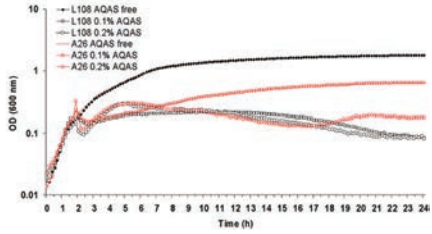

Fig. 3: Growth of AQAS selected mutant A26 and parent L108 at 37°C after inoculation with 4% vol/vol of overnight culture. AQAS was added to cultures at 2 h.

Virkon selected mutants V6 and V7 both grew significantly ($p > 0.01$) better than L354 when challenged with 0.5 X or the MIC of Virkon for L354 (figure 4).

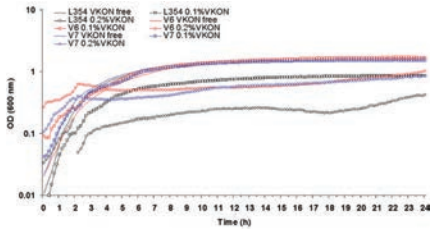

Fig. 4: Growth of Virkon selected mutants V6, V7 and parent L354 at 37°C after inoculation with 4% vol/vol of overnight culture. Virkon was added to cultures at 2 h.

No significant differences were observed between Farm Fluid S selected mutants F1 and F2 and their parent L357 in biocide free broth or when exposed to 0.5 X the MIC of Farm Fluid S. However both mutants grew significantly ($p > 0.05$) better when challenged with the MIC of Farm Fluid S (0.1% and 0.2% final concentration) as shown in figure 5.

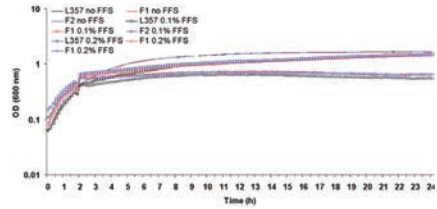

Fig. 5: Growth of Farm Fluid selected mutants F1, F2 and parent L357 at 37°C after inoculation with 4% vol/vol of overnight culture. Farm Fluid S was added to cultures at 2 h.

Table 1: Parent strains are in bold and mutants are listed below their respective parents. Mutants with increased resistance to biocide compared to their parents are indicated in red.

		MIC (%)			
Strain and Genotype	Selective agent	Virkon	Super-kill	AQAS	FFS
L357 (DT104 'A')		**0.4**	**0.025**	**0.1**	**0.2**
F1	FFS (1X MIC)				0.2
F2	FFS (1X MIC)				0.2
L378 (CipR VLA52)		**0.4**	**0.025**	**1.6**	**0.2**
A27				>3.2	
L108 (tolC::aph from SL1344)		**0.4**	**0.006**	**>0.003**	**0.025**
A26	AQAS (2X MIC)			0.12	
L358 (MDR DT104)		**0.4**	**0.025**	**1.6**	**0.2**
S2	S'kill (1X MIC)		0.025		
S22	S'kill (2X MIC)		0.025		
S23	S'kill (2X MIC)		0.025		
SL1344		**0.4**	**0.025**	**0.1**	**0.2**
V6	V'kon (2X MIC)	0.8			
V7	V'kon (2X MIC)	0.8			

Conclusion

This data clearly indicates that biocide exposure selects for strains with increased tolerance to biocides at sub-MIC concentrations and at the MIC and that there is no obvious fitness cost in these strains when compared to their parents in biocide free broth.

Growth of *Neisseria meningitidis* in a BMG LABTECH microplate reader with Atmospheric Control Unit (ACU)

Kerry L. Cutter
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- **Automated cell growth and culture studies using BMG LABTECH microplate readers equipped with ACU**
- ***Neisseria meningitidis* used to test Atmospheric Control Unit as it requires optimally 5% CO_2 to grow and thrive**
- **Improved growth rates observed in dilute cultures using the ACU as compared to using a CO_2 controlled incubator**

Introduction

It is well known that all organisms need a certain, but not necessarily a similar, level of carbon dioxide for growth and reproduction. The period during which this level is being increased corresponds to the lag phase as the organism is unable to divide until the critical concentration of CO_2 is reached.
The meningococcus has been considered one of the most fastidious micro-organisms with respect to growth requirements and it has long been recognized that most strains of *Neisseria meningitidis* require or benefit by, a concentration of CO_2 greater than atmospheric. Therefore, this organism is especially suitable for the study of CO_2 effect.
In this study, a strain of *Neisseria meningitidis* was used to assess the efficiency of a BMG LABTECH multimode plate reader coupled with an Atmospheric Control Unit (ACU) to deliver 5% CO_2. This was done by comparing growth of Neisseria meningitidis in the BMG LABTECH reader with a carbon dioxide incubator (set to deliver 5% CO_2) and in an incubator at atmospheric CO_2.

Materials & Methods

- BMG LABTECH microplate reader with ACU
- Lucy1, Anthos

Neisseria meningitidis C751 was grown on Brain-Heart Infusion (BHI) agar supplemented with 10% foetal bovine serum (FBS) at 37°C and in 5% CO_2 overnight.
The following day, several colonies were resuspended in 10ml BHI broth supplemented with 10% FBS. This was serially diluted to 10^{-6} and 200 µl samples of each dilution were dispensed in triplicate into a sterile 96-well microplate. Absorbance readings were taken of each well every hour (ABS filter 405nm) in the BMG LABTECH plate reader with ACU (set at 37°C, 5% CO_2) over a 24h time period.

Identical microplates were placed in a 37°C, 5% CO_2 incubator and a 37°C incubator with no supplemental CO_2, and absorbance readings (at 405nm) were taken at 0, 1, 2, 3, 4, 6, 8 and 24h using an Anthos Lucy 1 microplate reader.
Once absorbance readings had been collected for all treatments, the blank media control (values not shown) was used to adjust values to represent increase in OD. Percentage bacterial growth was then calculated using the maximum OD achieved for each treatment as 100%.
Data was taken from triplicate wells on two separate occasions to give six data sets for analysis. Microsoft Excel was used to plot graphs and Minitab 13 was used to carry out tests of statistical significance.

Results & Discussion

From the data shown in Figures 1-3 it is evident that there is a clear correlation between starting inocula and growth rate for all three treatments.
At the higher starting inocula there appears to be little difference in bacterial growth between treatments. This is most likely due to there being a sufficiently high number of organisms present to produce a critical level of CO_2, therefore enabling initiation of growth without the need for an external CO_2 source.
However, differences in growth rate of *N.meningitidis* C751 as a result of CO_2 effect are more apparent in the most dilute cultures. For example, when grown at 37°C in atmospheric CO_2 (figure 1), the most dilute culture (1 in 1,000,000 dilution) exhibits a prolonged lag period due to the inability of the small inocula to reach the critical level of CO_2 to initiate growth. This lag phase is appreciably shorter when the same culture is grown in either a carbon dioxide incubator (figure 2) or the BMG LABTECH plate reader with ACU achieving 29% and 72% bacterial growth respectively after 24 hours incubation.
By comparing the data collected using a carbon dioxide incubator and the new BMG LABTECH's ACU (figure 2) it can be seen that there is less difference between growth rates of the serial dilutions using the BMG LABTECH plate reader. This is most likely due to a constant temperature and level of CO_2 being maintained throughout the duration of the experiment. Whereas, when using a CO_2 incubator the samples have to be transferred to a microplate reader to measure absorbance resulting in both CO_2 level and temperature fluctuations which ultimately result in decreased growth rate of *N.meningitidis*.

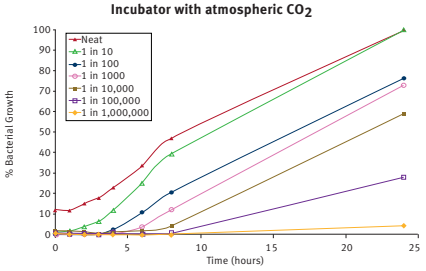

Fig. 1: Growth of serially diluted cultures of *Neisseria meningitidis* in BHI broth supplemented with 10% FBS at 37°C without supplemental CO_2. The data presented was calculated from triplicate optical density readings (at 405nm) taken in an Anthos Lucy 1 microplate reader from duplicate experiments.

Rev. 03/2007
Keywords: **ACU Atmospheric Control Unit, Gas vent, CO_2, Meningococcus, Microbial growth**

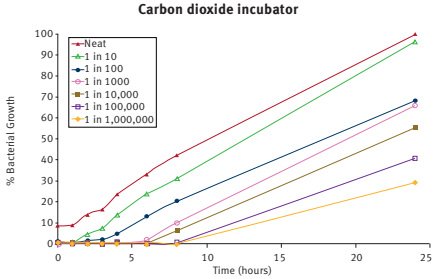

Fig. 2: Growth of serially diluted cultures of *Neisseria meningitidis* in BHI broth supplemented with 10% FBS in an incubator at 37°C, delivering 5% CO_2. The data presented was calculated from triplicate optical density readings (at 405 nm) taken in an Anthos Lucy 1 microplate reader from duplicate experiments.

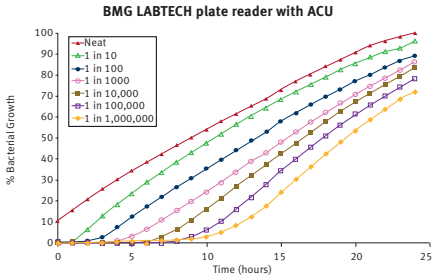

Fig. 3: Growth of serially diluted cultures of *Neisseria meningitidis* in BHI broth supplemented with 10% FBS using a BMG LABTECH plate reader with ACU set to deliver 5% CO_2 at 37°C. The data presented was calculated from triplicate optical density readings (at 405nm) taken hourly over a 24h period from duplicate experiments.

The results shown in Table 1 illustrate that this increased growth rate in the BMG LABTECH plate reader compared with a carbon dioxide incubator is statistically significant for all dilutions.

The results in Table 1 also reveal a significant difference in bacterial growth between the BMG LABTECH plate reader at atmospheric CO_2 for all except one dilution (1 in 10: p=0.052). However, the results also reveal that there is no significant difference in bacterial growth between a CO_2 incubator and atmospheric CO_2 which is most likely attributable to the temperature and CO_2 level fluctuations described earlier.

Table 1: Comparing growth of a serially diluted culture of *Neisseria meningitidis* C751 over a 24 hour period when grown at 37°C in a carbon dioxide incubator delivering 5%, in a BMG LABTECH plate reader with ACU delivering 5% CO_2 and with no supplemental CO_2 (atmospheric incubator). Minitab 13 was used to carry out t-tests and results were considered significant (+) or not significant (-) at the 95% confidence interval.

Comparison	CO_2 incubator vs BMG LABTECH	BMG LABTECH vs atmospheric incubator	CO_2 incubator vs atmospheric incubator
Dilution			
Neat	0.035 (+)	0.040 (+)	0.908 (-)
1 in 10	0.036 (+)	0.052 (-)	0.927 (-)
1 in 100	0.010 (+)	0.017 (+)	0.960 (-)
1 in 1,000	0.014 (+)	0.034 (+)	0.789 (-)
1 in 10,000	0.014 (+)	0.025 (+)	0.860 (-)
1 in 100,000	0.005 (+)	0.002 (+)	0.675 (-)
1 in 1,000,000	0.003 (+)	0.000 (+)	0.738 (-)

Conclusion

In conclusion, by utilizing the CO_2 dependency of a strain of *Neisseria meningitidis* it has been demonstrated that the BMG LABTECH plate reader with ACU is able to both achieve and sustain a level of CO_2 required for growth of such a fastidious organism. This study has also highlighted the advantage of this automated system over a carbon dioxide incubator. For example, conditions are more stable and the fact that it is less labour intensive means that a greater amount of data can be generated. This system is not only ideal for experiments using CO_2 dependent organisms such as *N.meningitidis* but may also prove very useful in cell culture studies.

Bradford assay performed on BMG LABTECH microplate readers

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E.J. Dell, BMG LABTECH, Durham, USA

- **Fast and homogeneous assay to determine the protein concentration of samples**
- **Dye shift can be followed with the spectrometer tool in the BMG LABTECH plate readers**
- **New easy to use BMG LABTECH evaluation software**

Introduction

Different colorimetric assays have been developed to determine the protein concentration of samples. The most commonly used methods are the Bradford assay, the Lowryassay and the BCA assay. In this application note we demonstrate how to determine the protein concentration of samples by using the Bradford assay and a BMG LABTECH microplate reader. The Bradford assay is based on the binding of protein to a dye, leading to a shift in the absorbance maximum of the dye. After creating a standard curve of protein solutions with known concentrations, the protein concentration of unknown samples can be calculated. The dye used for the Bradford assay is Coomassie® Brilliant Blue G-250 (Figure 1).

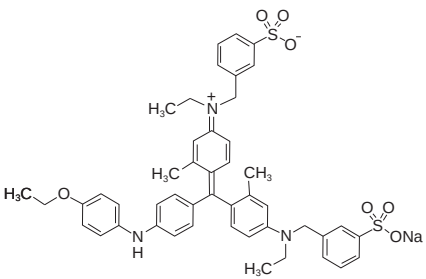

Fig. 1: Chemical structure of Coomassie® Brilliant Blue G-250.

Materials & Methods

- 96 well transparent microplates from Greiner
- BMG LABTECH microplate reader
- Bovine Serum Albumin from Sigma-Aldrich
- Bradford Reagent from Sigma-Aldrich

The Bradford Reagent was bought ready to use. A stock solution of bovine serum albumin in distilled water (10 mg/ml) was prepared as a protein standard. For the measurements, a dilution of bovine serum albumin was done starting with 1 mg/ml. Bradford reagent, 290 µl, was pipetted into a transparent 96 well microplate. 10 µl of the protein dilution was added followed by mixing in the wells. After 5 min of incubation at room temperature, the plate was read at 595 nm or in spectrum mode with the absorbance spectrometer of BMG LABTECH's microplate reader.

Instrument settings

Number of flashes:	20
Wavelength range:	380-800 nm (or discrete wavelength at 595 nm)
Wavelength step width:	1 nm

The acidic solution of the Coomassie® Brillant Blue dye has an absorbance maximum at 465 nm. After the addition of protein, hydrophobic amino acid residues and arginine residues bind to the dye. As a result, the absorbance maximum of the dye shifts from 465 nm to 595 nm (Figure 2).

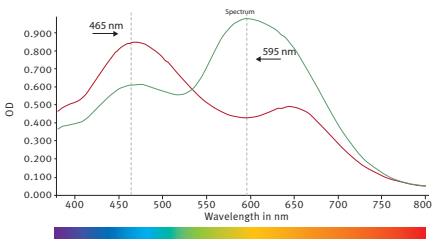

Fig. 2: The spectrum from unbound (red line) and protein bound (green line) Coomassie® Brilliant Blue. After binding the absorbance maximum of the dye shifts from 465 nm to 595 nm

The progress of the measurement can be followed using the Current State Window (Figure 3).

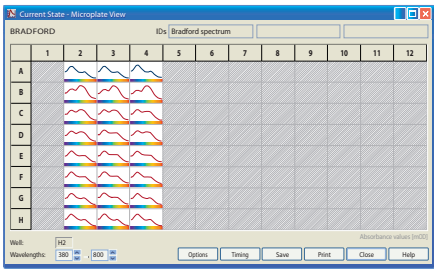

Fig. 3: Current State Window of Bradford measurements monitoring spectra from 380 to 800 nm. Standards are indicated as red lines, blanks are indicated as blue lines. Samples were run in triplicates.

Furthermore, during the measurement, it is possible to magnify a selected well and get information about the measured values over the spectral range (Figure 4).

Rev. 08/2007
Keywords: **A595, Bradford, Coomassie blue, Protein quantification, Spectral analysis**

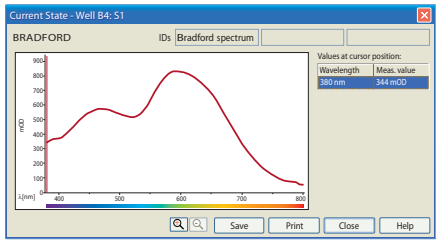

Fig. 4: Magnified current state picture of one selected well. The spectrum is taken from 380 to 800 nm. The cursor can be set to any wavelength for checking OD values during the measurement.

Results & Discussion

After measurements are taken, the data is transferred to the evaluation software. Pre-defined templates can be used to do the calculations needed instantaneously, i.e. average of raw data, blank correction, performing curve fits and much more.
For the Bradford assay the blank corrected values are used for the standard curve (Figure 5).

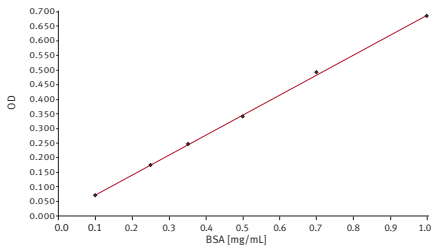

Fig. 5: BSA standard curve (linear regression fit performed with the new MARS Evaluation Software).

With the help of the standard curve the MARS data analysis software calculates the protein concentration for unknown samples automatically. If the option "path length correction" is used, the measured data is multiplied by a factor that depends on the type of microplate and volume used. With the help of this calculation, the data are normalized to a path length of 1 cm, thereby allowing a comparison to be made between absolute data obtained from a microplate reader with data obtained from a cuvette-based spectrometer.

Conclusion

The Bradford assay was successfully performed on the BMG LABTECH microplate reader. According to the manufacturers protocol this protein assay is linear in the range of 0.1 – 1.4 mg/ml. Because of its homogeneous and fast nature, the assay is a preferred method to determine the protein concentration of samples.

BMG LABTECH microplate readers offer entirely new possibilities with its spectrometer tool. A whole absorbance spectrum can be read in about 1 sec per well. Furthermore, the new MARS data analysis software allows for the absorbance maximum or minimum to be recognized at once after clicking on the spectral curve. Any wavelength can be selected to give the values for the optical density in any well. The speed of the spectrometer and the easy work-up in the software provide users with unmatched flexibility that can be used to optimize absorbance settings for all experiments.

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Comparison of thioredoxin activity in cortical neurons and glial cells using a BMG LABTECH microplate reader

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- **The thioredoxin system protects cells against H_2O_2-induced cell death**
- **Thioredoxin activity measured in neurons and glial cells using a BMG LABTECH microplate reader**
- **The thioredoxin activity of a neuron is 3.8 ± 0.86 fold higher than that of a glial cell**

Introduction

A carefully maintained intracellular redox equilibrium is necessary for the proper function of a living cell. Oxidative stress, or a disturbed intracellular redox balance, has been linked to several diseases, including neurodegenerative diseases such as Alzheimer's disease, rheumatoid arthritis, acute cerebrovascular disorders, diabetes and cancer, and accumulates in normal ageing. In oxidative stress there is increased production of oxidising species, primarily reactive oxygen species (ROS), which outperforms the ability of the intrinsic antioxidant defence mechanisms of the cell to neutralise them. Neurons are highly susceptible to oxidative damage due to high levels of ROS production through respiration and metabolism, presence of lipid peroxidation-susceptible polyunsaturated fatty acids in high concentrations and relatively low levels of some antioxidant enzymes, like catalase.

The main sulphur-centred antioxidant systems are the glutathione (GSH) and the thioredoxin (Trx)- reduction pathways. With both pathways, oxidative stressors such as hydrogen peroxide are reduced by NADPH, via a series of oxidations and reductions involving glutathione (GSH), Glutathione peroxidase (GPx) and Glutathione reductase (GR) for the former, and Peroxiredoxins (Prx) – or other proteins-, Thioredoxin (Trx) and Thioredoxin reductase (TrxR) for the latter. The GSH pathway is more effective in reducing small disulfide molecules and direct interactions with ROS, whereas Trx is more effective in repairing oxidised proteins by reducing their exposed disulfides while its own active site cysteine residues get oxidised (and reduced by TrxR).
The thioredoxin system protects cells against H_2O_2-induced cell death and its inhibition promotes oxidative stress, while thioredoxin-overexpressing mice display less oxidative brain damage following ischemia and live longer.

We are interested in thioredoxin activity in neurons and its potential changes under different conditions. However, because primary cortical cultures that we use as a model are mixed cultures of neurons and glial cells, we wanted to establish what the level of thioredoxin activity is in either cell type (neurons versus glia).

Materials & Methods

All materials were obtained through normal distribution channels from the manufacturers stated.

- Transparent 96-well plates (Greiner)
- BMG LABTECH microplate reader
- Leica DMI6000B inverted epifluorescence microscope

Neuronal cultures and trophic deprivation

Cortical rat neurons were cultured as described from E21 rats. Glial cells were cultured by plating rat cortical homogenate at half the density of that for neuronal cultures and grown in DMEM (Dulbecco's Modified Eagle's Medium) including 10% Fetal Calf Serum (Invitrogen) and antibiotics (Penicillin/Streptomycin 1:100, Sigma). No AraC (Cytosine β-D-arabino-furanoside hydrochloride, a DNA synthesis inhibitor, Sigma) was added to glial cultures in contrast to neuronal ones at DIV4 (Day In Vitro 4). Neurons were killed in glial cultures by overnight incubation in 1 mM NMDA (N-methyl-D-Aspartic acid, Calbiochem) in minimal medium (TMo) on DIV7. Trophic deprivation was done in both types of cultures after a culturing period of 8-10 days during which cortical neurons develop a network of processes, express functional NMDA-type and AMPA/kainate-type glutamate receptors, and form synaptic contacts. Cells were transferred into defined medium lacking trophic support, hereafter "TMo": 10% MEM (Invitrogen), 90% Salt-Glucose-Glycine (SGG) medium (SGG: 114 mM NaCl, 0.219% NaHCO3, 5.292 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 10 mM HEPES, 1 mM Glycine, 30 mM Glucose, 0.5 mM sodium pyruvate, 0.1 % Phenol Red; osmolarity 325mosm/l).

Thioredoxin Activity Assay

The thioredoxin "insulin-reducing assay" was performed with some modifications. Briefly, cells were grown for 24 h in minimal medium TMo, then lysed by scraping on ice in 150 µl lysis buffer/35 mm dish (20 mM HEPES pH 7.9, 100 mM KCl, 300 mM NaCl, 10 mM EDTA, 0.1 % Triton X-100, 1:100 Protease Inhibitor Cocktail III- Calbiochem) after being washed twice in pre-warmed TMo medium. Cell lysates were collected in microcentrifuge tubes and cell debris discarded by centrifugation for 3 min at 10000 rpm at 4 °C. The appropriate volume of lysate containing 30 µg protein was diluted in lysis buffer to a final volume of 34 µl. (We tested a series of protein concentrations to select one that fell within the linear range of the reaction, starting with amounts of protein that had been shown to be in the linear range for this assay previously. Alternatively or in addition, a standard curve using purified thioredoxin can be included to confirm the linear range of the assay.) 1 µl DTT activation buffer (50 mM HEPES pH 7.6, 1 mM EDTA, 1 mg/ml BSA, 2 mM DTT) was added to all tubes. After mixing, the tubes were incubated at 37°C in a waterbath for 15 min, for reduction of endogenous thioredoxin. 20 µl reaction mixture (200 µl 1M HEPES pH 7.6, 40 µl 0.2M EDTA, 40 µl NADPH 40 mg/ml and 500 µl insulin 10 mg/ml) was then added to each tube, as well as 0.5 units of Trx reductase or H_2O for negative controls, and the samples were incubated at 37°C for 20 min. The reaction was stopped by addition of 250 µl Stop Buffer (6 M guanidine HCl, 1 mM DTNB

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Keywords: **Cell death, Glial, H_2O_2, Neurons, Thioredoxin**

(Sigma) in 0.2 M Tris-HCl pH 8.0), 200 µl of sample were transferred to a 96-well plate and absorbance was measured at 405 nm in a BMG LABTECH microplate reader.

Assay Principle

(1) $Trx\text{-}S_2 + DTT_{red} \rightarrow Trx\text{-}(SH)_2 + DTT_{ox}$
(2) $Trx\text{-}(SH)_2 + Insulin\text{-}S_2 \rightarrow Trx\text{-}S_2 + Insulin\text{-}(SH)_2$ TrxR
(3) $Trx\text{-}S_2 + NADPH + H+ \rightarrow Trx\text{-}(SH)_2 + NADP^+$
(4) $Insulin\text{-}(SH)_2 + DNTB \rightarrow Insulin\text{-}S_2 + 2TNB_{(Bright\ yellow)}$

Endogenous Thioredoxin (Trx) in the samples is first reduced by dithiothreitol (DTT, $_{red}$ for reduced and ox for oxidised forms); (reaction 1). After the addition of NADPH, Insulin and Thioredoxin Reductase (TrxR), Trx reduces insulin disulfides (reaction 2). In the presence of TrxR and at the expense of NADPH, Trx can be reduced and brought back into the system (reaction 3). When the reaction is stopped the sulfhydryl groups are derivatized to TNB (reaction 4) which gives an intense yellow colour.

Immunocytochemistry and cell counting

Cell cultures were fixed with 3 % paraformaldehyde and 4 % sucrose for 20 minutes at room temperature (RT) and then permeabilized for 5 min in phosphate-buffered solution (PBS) supplemented with 0.5 % NP-40. Cells were incubated overnight at 4°C in PBS with primary antibodies against glial fibrillar acidic protein (GFAP) (1/2000; Sigma) and Neuronal Nuclei (NeuN; 1/15; Chemicon), to stain glial cells (astrocytes) and neurons, respectively. NeuN staining was completed with 1 h incubation with biotinylated anti-mouse (1/200); (Jackson ImmunoResearch) antibody, followed by 1 h incubation with Cy3-coupled streptavidin (1/500).
GFAP staining was completed with Alexa 488-coupled anti-rabbit (1/500) antibody (Jackson ImmunoResearch) incubated for 1 hour at RT. After mounting with DAPI-containing solution (Vectashield; Vector Laboratories), cells were observed and images were taken using the 10X or 20X objective of a Leica inverted epifluorescence microscope driven by LAS software. Cells were counted (blind) to establish the total number of cells per culture type (DAPI-stained) and the percentage of NeuN-positive and GFAP-positive cells, using ImageJ software.

Results & Discussion

We performed thioredoxin activity assays in parallel in pure glial cell cultures and mixed neuronal-glial cultures (thereafter referred to as "cortical") that were under trophic deprivation for 24 h. Absorbance was measured in a BMG LABTECH microplate reader and a control reaction without Thioredoxin reductase enzyme was performed for each sample and this value was subtracted from the value of the sample containing TrxR.

At the same time, we used immunostaining to establish the number of cells present in each type of culture.
From the thioredoxin activity assay data combined with the cell counts from parallel dishes, the thioredoxin activity of a neuron in a mixed cortical culture, relative to the activity of a glial cell was calculated using the formula: "(number of glial cells/cort. dish)*Glial activity + (number of neuronal cells/cort.dish)*Neuronal activity = (fold-increase of Trx activity in cortical compared to glial culture)*(number of glial cells/glial dish)*Glial activity".
Thus, the thioredoxin activity of a neuron is 3.8 ± 0.86 fold higher than that of a glial cell (Figure 1). Since the glial cells in a mixed cortical culture represent 4-9 % of the total number of cells, the thioredoxin activity represented by glial cells is only 1-2.5 % of the total thioredoxin activity in that culture.

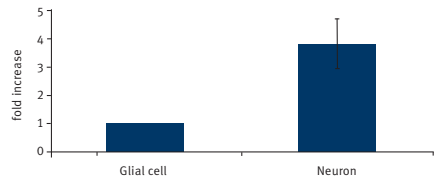

Fig. 1: Relative thioredoxin activity of neurons versus glial cells. n = 4.

Conclusion

In our system only 1-2.5 % of the thioredoxin activity in primary cortical cultures is attributable to glial cells, even though glial cells represent 4-9 % of the total cell number. Therefore, neurons are responsible for the vast majority of thioredoxin activity in mixed rat cortical cultures. The thioredoxin activity assay can easily be performed on a BMG LABTECH microplate reader.

Reaction optimization by parallel kinetic studies with the FLUOstar® Omega

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- **Kinetic studies on the metallation of porphyrin**
- **Changes in the absorbance spectra monitored with the FLUOstar® Omega**
- **Relative reaction rate in twelve different solvents determined in parallel**

Introduction

The yields of chemical reactions may vary considerably with parameters such as solvent, catalyst, temperature and reaction time. A challenge for the chemist is to optimize these parameters to obtain a satisfactory yield at acceptable time and cost. To aid optimization, a number of devices are available that increase the number of reactions that can conveniently be performed in parallel by an individual chemist.

In situations where reactants and products differ in their molar extinction coefficients the reaction progress can be followed directly by UV-visible spectroscopy. Rather than determining the concentrations of species by physical separation and individual quantitation, a time-series of spectra in which these species vary in their relative proportions can be analysed mathematically accompanied by derivation of kinetic rate constants by least squares fitting.

Devices such as the Radleys carousel reactor allow 12 reactions to be performed simultaneously on one standard hot-plate stirrer. Reaction aliquots sampled as a function of time are therefore readily accommodated in the 12 columns of a 96-well microplate. Monitoring is ideally suited to the BMG LABTECH FLUOstar Omega microplate reader due to its ability to acquire a full UV-visible spectrum of each well in under one second.
In this example we have monitored the metallation of a porphyrin with Zn^{2+} (Figure 1) as a function of solvent. The porphyrin starting material and product are both highly coloured and the reaction proceeds with a perceptible but subtle colour change. The absorption spectra of the two species overlap significantly making a global least squares analysis of spectra desirable for quantitative rate comparisons.

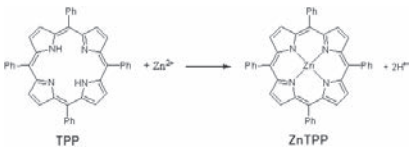

Fig. 1: Metallation of tetraphenylporphyrin (TPP) with zinc.

Materials & Methods

- BMG LABTECH FLUOstar Omega microplate reader
- Corning Costar flat bottom polypropylene 96-well microplates
- Tetramethylrhodamine methyl ester (TMRM), Sigma
- Radleys Carousel 12 Reaction Station, with magnetic stirrer
- Specfit/32 software (Spectrum Software Associates)

5,10,15,20-Tetraphenylporphyrin (TPP) was prepared by the method of Adler et al. and recrystallized from chloroform layered with methanol. Solvents (N,N-dimethylformamide (DMF), toluene, chloroform, pyridine, isopropanol, acetone, N-methyl-2-pyrrolidone (NMP), dichloromethane, acetonitrile, tetrahydrofuran, ethylacetate and dimethylsulfoxide) were obtained from Acros or Aldrich.
Background scans of all wells of a microplate were made on the FLUOstar Omega using the UV/Vis absorbance spectrometer (20 flashes per well, 450–680 nm with 1 nm resolution). TPP (2 mg) was placed with a stirrer bar in each of the 12 tubes of the carousel reactor. Solvent (10 mL) was added to each tube, which was stirred for ~15 minutes to allow dissolution of the porphyrin. Aliquots (50 µL) were removed from each tube and diluted with DMF (200 µL) in columns 1-12 of row A of the microplate, which was immediately scanned.
Zn(OAc)2.2H2O (obtained from Avocado) portions were weighed (12 × 7 mg), then one portion added in quick succession to each carousel tube to initiate the reaction. After total times of 10, 30, 60, 120, 240, 480 and 1440 minutes, aliquots (50 µL) were withdrawn from each tube, diluted with DMF (200 µL) in a microplate row and scanned immediately.
The empty plate background was subtracted and the absorbance at 680 nm set to zero for each scan by subtracting a constant offset at all wavelengths. The data were subjected to singular value decomposition using the Specfit/32 software, followed by kinetic modelling according to the scheme TPP → ZnTPP where both are coloured species with first order kinetics with respect to TPP. The Zn^{2+} concentration remains approximately constant due to the ten fold excess used, and no significant difference in the relative rates or quality of fit was observed if the reaction was modelled as TPP + Zn^{2+} → ZnTPP, with colourless Zn^{2+} and first order with respect to both TPP and Zn^{2+}.

Results & Discussion

The rate of the metallation reaction of tetraphenylporphyrin (TPP) with Zn^{2+} (Figure 1) might show solvent dependence, due to differing degrees of solvation of the porphyrin and zinc salt and the possibility for bases to assist in porphyrin deprotonation. In preliminary experiments polystyrene microplates were found to be attacked too rapidly by most organic solvents, so we investigated the possibility of using flat bottom polypropylene storage plates as an alternative to costly and fragile quartz. At the visible wavelengths of interest,

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Keywords: **Catalyst, Chemical reaction optimization, Metallation, Solvent, Spectral analysis**

these plates proved to be sufficiently transparent, although it was found necessary to collect a background scan of each well of the empty plate due to well-to-well variation.

Twelve common laboratory solvents spanning a range of polarity and chemical properties were chosen for comparison. Starting material and product were both found to dissolve adequately in DMF. The reaction progress could be qualitatively assessed by visual inspection of spectra (Figure 2 and 3) using the MARS evaluation software, which revealed most rapid reaction in the halogenated solvents dichloromethane and chloroform and little change in N-methyl-2-pyrrolidone (NMP).

Isopropanol and acetonitrile reactions gave lower maximum OD values than the other samples which can be attributed to poor solubility of the TPP starting material in these solvents. For DMSO a black precipitate was apparent in the sampled reaction aliquot, possible evidence of a side reaction.

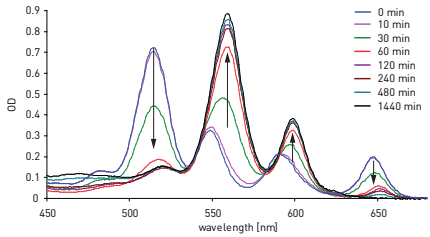

Fig. 2: Changes in visible spectrum accompanying zinc metallation of TPP in chloroform. Arrows indicate the evolution of the absorption bands with time.

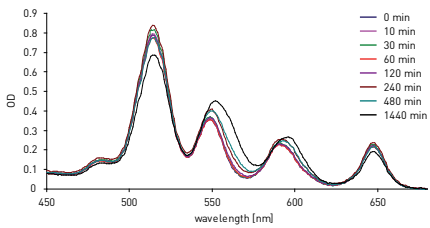

Fig. 3: Changes in visible spectrum accompanying zinc metallation of TPP in NMP.

To quantify the relative reaction rates in the different solvents, spectra were analysed using the Specfit/32 software. Spectra were subjected to singular value decomposition and globally fitted to a kinetic model in which the only coloured species were TPP and ZnTPP. The reaction was assumed to be first order with respect to TPP. There was no evidence from the factor analysis of more than two coloured species, and we were unable to observe the presence of an intermediate that had been suggested in the literature. Acceptable fits to the model were obtained for all solvents (estimated errors <20%), with the exceptions of acetonitrile, isopropanol and DMSO. The relative rates deduced from this analysis are given in Table 1.

Acetonitrile and isopropanol are excluded due to insolubility of the TPP starting material, and the value for DMSO should only be taken as approximate.

Table 1: Rates of reaction of tetraphenylporphyrin with $Zn(OAc)_2.2H_2O$ in different solvents, relative to the rate in N-methyl-2-pyrrolidone (NMP).

Solvent	Relative initial rate	Solvent	Relative initial rate
DMF	8	NMP	1
Toluene	10	Dichloromethane	300
Chloroform	100	Tetrahydrofuran	30
Pyridine	2	Ethylacetate	30
Acetone	50	Dimethylsulfoxide[a]	~1

The metallation reaction is fastest in the two halogenated solvents, dichloromethane and chloroform. The rates in the highly polar solvents NMP, DMF and DMSO are amongst the slowest. The basic and aromatic solvent pyridine, was less effective than non-basic toluene and two orders of magnitude slower than dichloromethane.

Conclusion

A carousel reactor could be used in conjunction with the FLUOstar Omega to conveniently monitor the kinetics of twelve reactions in parallel. Polypropylene microplates were found to be compatible with measurement of visible spectra of organic solutions, and sampling and scanning of the twelve reactions could be achieved in three minutes.

The ability of the FLUOstar to collect complete spectra enabled the use of singular value decomposition and global least squares fitting to analyse reaction kinetics.

Enzyme kinetic measurements performed on a BMG LABTECH microplate reader

Franka Ganske
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- **Esterase activity determined using the p-nitrophenyl acetate (pNPA) assay**
- **K_m and V_{max} calculated using new features in the MARS evaluation software**
- **Michaelis-Menten, Lineweaver-Burk, and other plots are presented**

Introduction

Esterases catalyze hydrolysis reactions by converting esters into an acid and an alcohol using water as nucleophil. Therefore, esterases belong to the enzyme group of hydrolases. They are often used as biocatalysts to produce optically pure compounds.
Esterases differ in their affinity to specific substrates and this affinity is represented by the Michaelis-Menten constant K_m. It describes the affinity of an enzyme to a substrate. K_m is equivalent to the substrate concentration at which the reaction velocity is half of the maximal velocity. That means that a high affinity to a substrate leads to a small K_m-value and vice-versa.
BMG LABTECH has developed a new evaluation software feature able to calculate the K_m value as well as the maximal velocity (V_{max}) from an enzymatic kinetic measurement. Next to the Michaelis-Menten fit, other linearized fits are also shown and a comparison of the results is given.
As a model reaction, the p-nitrophenyl acetate (pNPA) assay was performed on a BMG LABTECH microplate reader.

Assay Principle

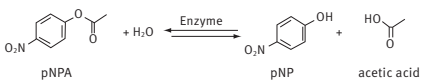

Fig. 1: Scheme of the pNPA assay.

The enzyme hydrolyzes the acetate ester with the help of water. The products are acetic acid and p-nitrophenol (pNP), the latter showing an absorption maximum at about 405 nm.

Materials & Methods

- p-nitrophenol (pNP) and p-nitrophenyl (pNPA) acetate, Sigma Aldrich
- Clear 96-well microplates, Greiner
- BMG LABTECH microplate reader
- MARS Data Analysis Software, Version 1.20 or higher
- Two different enzyme preparations (E1 and E2) were kindly provided by the working group of Prof. Bornscheuer, Greifswald

Enzyme kinetic measurements

First a 10 mM stock solution of the substrate pNPA in DMSO is prepared. In each well 190 µL phosphate buffer (50 mM, pH 7.4) and 10 µL enzyme preparation is pipetted. 40 µL of substrate (solved in DMSO) at different concentrations is added using the onboard injectors. The final volume per well is 240 µL. The enzymatic reaction starts after adding the substrate. The absorbance is measured at 410 nm for every second for 90 seconds at 37°C. The blank represents the autohydrolysis of pNPA without enzyme. In addition, a pNPA-free negative control (NC) is run that consists only of buffer and enzyme.
The measurement is performed using the well mode (see instrument settings below) that provides the possibility to inject and to measure absorbance at the same time.

Instrument settings

Measurement type:	Absorbance, Well Mode
No. of intervals:	90
Kinetic interval time:	1 s
No. of flashes per well:	20
Positioning delay:	0.5 s
Target temperature:	37°C
Filter:	410-10 nm

Injection settings:

Volume of pump 1 and 2:	individual
Pump speed:	310 µl/s
Injection start time:	2.0 s

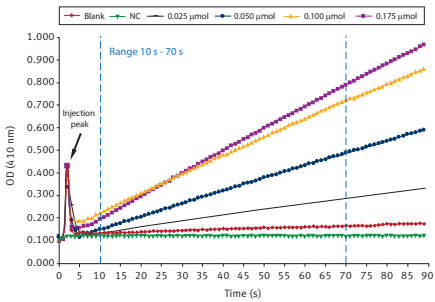

Fig. 2: Signal curves of esterase-catalyzed reactions using different concentrations of the substrate pNPA (pNPA concentrations range from 0.025 µmol to 0.175 µmol / 240 µL).

pNP standard curve

For the calculation of the initial reaction rates it is necessary to convert the delta OD values per minute into µmol product per minute. Therefore a standard curve of the product using the same reaction conditions as for the kinetic measurements has to be done. From a 10 mM pNP stock solution, solved in phosphate buffer (50 mM, pH 7.4), different dilutions of pNP ranging from 0.0025 µmol to 0.1 µmol were prepared in 200 µL. 40 µL of DMSO were added simulating the DMSO concentration in the kinetic samples. The optical

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Keywords: **Enzyme kinetics, Esterase, Injectors, K_m, Michaelis Menten, V_{max}**

density (OD) is measured using an absorbance 410-10 nm filter. Figure 3 shows a pNP standard curve.

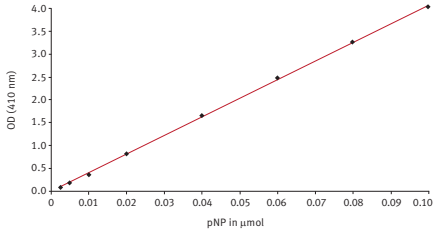

Fig. 3: pNP standard curve using 240 µL volume with 16.6 % DMSO.

The slope of the standard curve stands for the extinction coefficient for pNP under the conditions mentioned. It was determined to be about 41 OD per µmol. The coefficient is used to calculate the initial reaction velocities.

Results & Discussion

After the enzyme kinetic measurement is finished, the evaluation software provides different plots and presents the corresponding K_m and V_{max} values. The Michaelis-Menten fit shows the reaction velocities depending on the substrate concentration (Figure 4).

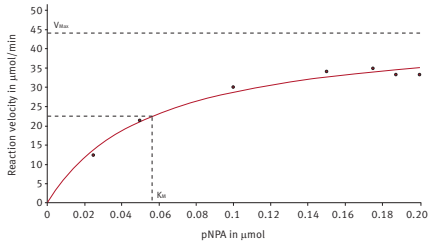

Fig. 4: Michaelis-Menten plot of the reaction velocity, obtained with the E1 enzyme preparation, depending on the substrate concentration. K_m is the substrate concentration corresponding to the half of the maximal velocity.

Next to the Michaelis-Menten fit different linearized plots are available.

Figure 5 shows such a linearized diagram (Hanes plot).

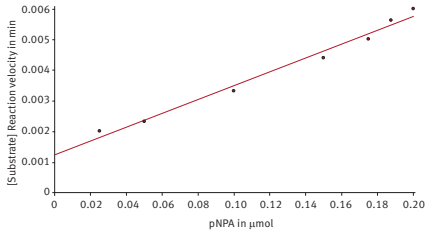

Fig. 5: Plot of an E1 reaction. The ratio of the substrate concentration and the velocity is presented depending on the substrate concentration. The reverse of the slope represents V_{max}. The intersection of the y-axis represents K_m/V_{max}.

The results of further linearized fits are compared to the Michaelis-Menten findings in table 1.

Plot	E1		E2	
	K_m [µmol]	V_{max} [µmol/min]	K_m [µmol]	V_{max} [µmol/min]
Michaelis-Menten	0.056	44.8	0.12	16.3
Lineweaver-Burk	0.073	49.2	0.08	12.5
Eadie-Hofstee	0.063	46.4	0.07	13.2
Scatchard	0.068	47.9	0.10	15.2
Hanes	0.056	44.4	0.11	15.5

Table 1: K_m and V_{max} comparison obtained from different enzyme kinetic plots.

The different methods to determine K_m and V_{max} have all assets and drawbacks. Because of that it is recommended to have a look at different plots for deciding which calculated constants are really correct. With the help of the MARS Data Analysis software enzyme kinetic evaluation is very simple because every plot is available directly after measurement and calculation.

Conclusion

The new software feature for enzyme kinetic offers fast and easy calculation of K_m and V_{max}. Available plots are the common Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee, Scatchard and Hanes kinetic fits.

High-throughput method for dual assessment of antifungal activity and growth kinetics using a FLUOstar® Omega

Gordon Ramage, Ryan Quinn and Steven Milligan
Section of Infection and Immunity, Glasgow Dental School, Faculty of Medicine, University of Glasgow, UK

- **Effect of tea tree oil on yeast growth measured on the FLUOstar® Omega**
- **Minimal inhibitory concentration of antifungal agents determined kinetics**
- **Growth and inhibitory effects can be assessed in tandem**

Introduction

High throughput testing is an essential requirement for testing panels of potential antimicrobial molecules against clinically important pathogens. This is particularly important considering there is an increasing incidence of infectious diseases and dwindling arsenal of efficacious antimicrobial agents. The pharmaceutical industry, commercial laboratories and independent university researchers therefore require sensitive tools to enable them to detect antimicrobial activity from their chosen panel of molecules.

Current methodologies largely rely on endpoint broth microdilution or agar diffusion to assess antimicrobial activity. Assessing antimicrobial activity using endpoint readings is difficult, as this relies on a visual inspection of turbidity and observing well within a microtitre plate with little or no growth in comparison to the untreated control. Although the absorbance of the endpoint assay can be read spectrophotometrically, this does not provide accurate data relating to the kinetics of inhibition, which may be subtle in many instances due to slow growth rates. For example, antimicrobial activity may be influenced by the rate of growth in a particular media, e.g. Mueller Hinton broth, which again cannot be accurately assessed by an endpoint visual inspection. Therefore, an accurate interpretation of the data is not possible.

The experimental approach described within this application note aimed to determine the ability of the FLUOstar Omega to accurately quantify the inhibitory properties of the novel antimicrobial agent tea tree oil (TTO) grown against the pathogenic yeast *Candida albicans*. The results are compared to growth rates when the known antimicrobial agent Amphotericin B is present.

Materials & Methods

- Clear polystyrene 96-well plates (Corning)
- Melaleuca alternifolia tea tree oil (Sigma-Aldrich)
- FLUOstar Omega (BMG LABTECH)
- Amphotericin B (AMB (Sigma-Aldrich))

Candida albicans was propagated overnight in yeast peptone dextrose (YPD) broth at 37°C on an orbital shaker. The resultant yeast suspension was washed in PBS by centrifugation and counted in a haemocytometer. The yeast cells were standardised to approximately 1×10^6 cells per millilitre and then diluted one hundred fold into fresh YPD broth. TTO and AMB were then prepared in YPD containing 0.05% v/v Tween 80 at 0.01, 0.05, 0.1 and 0.5% for TTO and 0.0002, 0.0001, 0.00005 and 0.000025% w/v for AMB. Yeast cells were diluted into each TTO suspension to a final concentration of 1×10^4 cells/mL, then 200 µL volumes arrayed in quadruplicate into adjacent columns of a 96-well microtitre plate. A negative control without any reagent was included. The plate was placed inside the FLUOstar Omega with the incubator preset to 37°C. The absorbance mode was set to read at 550 nm with orbital shaking for 30 seconds prior to each read. This was programmed to take forty eight individual measurements over a 24 hour period.

Results & Discussion

With increasing concentrations of TTO relative to the untreated control, a significant reduction of growth is observed (Figure 1).

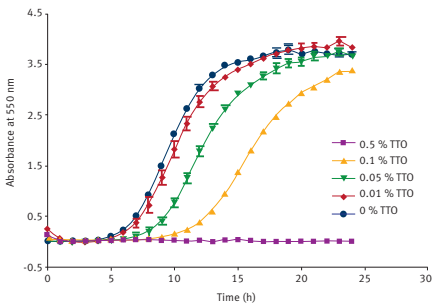

Fig. 1: Dose dependant growth curves of *Candida albicans* in TTO. The mean of the quadruplicate data was taken and standard error bars presented. The data was imported and presented using GraphPad Prism software.

In addition, this data demonstrates dose dependant inhibitory characteristics, which would be impossible to differentiate from a 24 endpoint read. This point is clearly demonstrated when the absorbance data from the 12 h and 24 h time points are compared to the untreated control (Fig 2). After 12 h incubation, significant differences are observed between the various concentrations tested, whereas at 24 h incubation the difference in mean absorbance are minimal.

Rev. 03/2009
Keywords: **Antifungal agents, *Candida albicans*, Microbial growth, Tea tree oil, Yeast**

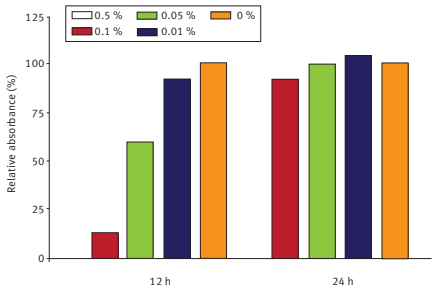

Fig. 2: Relative absorbance of *Candida albicans* at 12 and 24 h in TTO. The mean of data points at 12 and 24 h from each concentration tested were calculated, and the treated cells were compared relatively to 12 and 24 h controls (100%).

Following studies on the novel antifungal TTO, the well described and potent antifungal agent AMB was added as described above at different concentrations to Candida albicans suspensions (Fig 3). The data showed that AMB exhibited a clear inhibitory profile at the highest concentrations (0.0002%), with a reduced growth rate at 0.0001%. However, at all other concentrations examined the growth rates were similar to AMB free cell suspensions, indicating that the concentration of AMB is critical in inducing cell membrane instability.

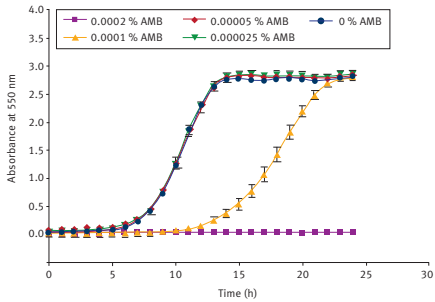

Fig. 3: Dose dependant growth curves of *Candida albicans* in AMB. The mean of the quadruplicate data was taken and standard error bars presented.

Overall these data demonstrate that the antifungal mode of action is an important determinant of the inhibitory and growth profiles exhibited by *Candida albicans*. AMB is a polyene antifungal agent that disrupts cell membranes and allows cytoplasmic components leak out causing cell death, which is induced through specific interaction with ergosterol in the cell membrane. TTO is also proposed to act on the cell membrane, but as this is comprised of a complex mixture of terpinenes, its specific mode of action has yet to be determined. It is likely to alter membrane permeability in a non-specific and concentration dependant manner, which is demonstrated from the pattern of growth kinetics obtained in the experiments described herein.

Conclusion

In this experiment we show that minimum inhibitory concentration assays can be assessed using the FLUOstar Omega to provide a sensitive and accurate analysis of growth kinetics to demonstrate subtle dose dependant drug effects that otherwise would be missed using endpoint readings. The benefit to this method for screening is that *Candida albicans* and other pathogenic microorganisms can be tested with a range different antimicrobial agents and concentrations.

Furthermore, if different fluorescent strains are available, both growth and inhibitory properties of mixed microbial populations can be assessed in tandem.

High-throughput monitoring of bacterial growth at elevated hydrostatic pressure on a BMG LABTECH microplate reader

S. Lucas Black[1,2], F. Bruce Ward[1] and Rosalind J. Allen[2]
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- **Development of a microplate sealing technique suitable for growth of microorganisms at high hydrostatic pressure**
- **High-throughput monitoring of growth of the deep sea bacterium *Photobacterium profundum* SS9**
- **The measured growth rates are dependent on both the hydrostatic and osmotic pressure**

Introduction

Microorganisms display an astonishing ability to survive and proliferate under extreme environmental conditions, including high and low temperatures, high acidity or alkalinity, high salt concentrations, and high hydrostatic pressure. The physiological and biochemical origins of these capabilities are in many cases poorly understood. Technologically, extremophiles have the potential to provide new products for molecular biology and other biotech areas.

Our work focuses on the deep sea bacterium *Photobacterium profundum* SS9. This γ- proteobacterium is a close relative of *Vibrio cholerae*. *P. profundum* SS9 was isolated in 1984 from the Sulu Sea at a depth of 2.5 km, and is a piezophile: it grows better at elevated hydrostatic pressure than at atmospheric pressure. *P. profundum* SS9 can grow at pressures ranging from 0.1 MPa to 90 MPa, with an optimal growth pressure of 28 MPa. Like most marine organisms, *P. profundum* SS9 has a requirement for NaCl. It has been observed that the physiological effects of increased hydrostatic pressure are similar to those of osmotic pressure (increased salt). For example, *P. profundum* SS9 produces a similar range of intracellular osmolytes in response to both salt and hydrostatic pressure. The aim of our work is to understand better this intriguing similarity.

Fig. 1: The 3 litre pressure vessel used in this study.

This study requires us to grow *P. profundum* SS9 under a range of conditions of hydrostatic and osmotic pressure in the lab. BMG LABTECH microplate readers are extremely valuable for such high-throughput growth studies, as they allow fast and reliable measurements to be achieved much less labour intensively than using traditional methods, and allow much better statistics to be obtained through the use of multiple replicates.

Growth of microorganisms is monitored by measuring the absorbance at 600 nm (A_{600}) in the plate reader over time. However, the use of a microplate reader for growth under hydrostatic pressure provides unusual challenges. Growth of microorganisms at pressure is achieved using a pressure vessel. The steel pressure vessel used in this work is pictured in Figure 1; it is filled with liquid water which is pressurized. The vessel can accommodate up to 8 standard sized microplates. These microplates must be sealed with a film, attached by a strong adhesive, to prevent leakage in the pressure vessel. The sealing film must be flexible to allow pressure to be transmitted to the well contents. The wells must not contain any air, as gas pockets in the wells will be compressed on pressurization, causing the seal to stretch and break. Furthermore, the seal must be transparent to allow absorbance measurements, and must be non-toxic to the microorganisms under study.

We have developed a method for sealing microplates that overcomes these challenges. In this application note, we report preliminary results using this method in combination with a BMG LABTECH microplate reader.

Materials & Methods

Bacterial Strains and Media

Photobacterium profundum SS9 was obtained from Prof. Doug Bartlett (Scripps Institute of Oceanography) and cultured in modified MOPS Minimal (MM) Media. The MM media was made according to Neidhardt et al. with a final concentration of 40 mM MOPS, 25 mM Glucose, 9.5 mM NH_4Cl, 4 mM Tricine, 1.32 mM K_2HPO_4, 0.525 mM $MgCl_2$, 0.276 mM K_2SO_4, 0.05 mM $CaCl_2$, 0.01 mM $FeSO_4$ and NaCl concentrations ranging from 100 mM to 700 mM. In addition, trace elements and vitamin solutions are added, along with 0.1% casamino acids.

Preparation of Pressure Proof 96-well Plates

Each well of a Greiner Bio-One flat bottom 96-well plate was filled with 382 µL liquid media. The plate was then sealed with a commercial PCR film, ensuring that air bubbles were not trapped beneath the film. Once the film was in place, a layer of araldite epoxy resin was spread around the edge of the film and allowed to set. This is necessary to prevent leakage in the pressure vessel.

Culturing Bacteria at High Pressure and Calculation of Growth Rate

High pressure growth at 28 MPa was carried out in a 3 litre pressure vessel with a temperature controlled water jacket set to 15°C. Pressure was generated by a hand pump. To make a measurement of A_{600}, the microplates were depressurized, dried and A_{600} was

Rev. 03/2009
Keywords: **Bacterial growth, Extremophile, Hydrostatic pressure, OD_{600}, Piezophile**

measured in the BMG LABTECH microplate reader, without removing the seals. Measurements were made at 3 hour intervals for 81 hours.

Each experiment was performed using three identical 96-well microplates. Each plate contained 11 replicates of 7 different NaCl concentrations. For each NaCl concentration, a blank well contained media alone. The final 12 wells per plate were made up of 4 replicates of 3 crystal violet solutions of known A_{600} ranging from 0.1 to 1.5.

Measurements at different time points were merged using the MARS data analysis software from BMG LABTECH. This data was then exported into Microsoft Excel. During the experiment, the A_{600} of our blank wells changed over time: this is because the PCR film reacts slowly with the culture medium and becomes slightly opaque. To correct for this effect, we subtracted from each raw data point the measured A_{600} for the blank well (at that NaCl concentration), measured at the same time. This method of blanking was tested using the 12 wells per plate containing crystal violet solutions of known A_{600}; the measured A_{600} increased with time in a linear fashion. From the corrected A_{600} data, we were able to determine the growth rate of *P. profundum* SS9 under a range of NaCl concentrations, by taking the slope of a plot of log (A_{600}) versus time, for the exponential part of the growth curve.

Results & Discussion

The experiment was carried out at 28 MPa (optimal growth pressure for *P. profundum* SS9) and at 0.1 MPa (atmospheric pressure). We also carried out two control experiments. In the first control, the microplate was not sealed and growth was monitored at atmospheric pressure, with the aim of determining whether oxygen availability in the sealed plates inhibited the growth of *P. profundum* SS9. In the second control, also at atmospheric pressure, a standard polystyrene microplate lid was used and gas exchange with the wells was prevented by taping the lid in an anaerobic chamber. This should result in the same growth rate as our sealed microplate method (at atmospheric pressure) and thus acts as a control for our method.

Our results are shown in Figure 2. Hydrostatic pressure has a marked effect on the range of NaCl concentration at which *P. profundum* SS9 is able to grow. When the hydrostatic pressure was increased from 0.1 MPa to 28 MPa, growth of *P. profundum* SS9 was largely unaffected at intermediate salt concentrations (200-400 mM NaCl), but growth was strongly inhibited at higher NaCl concentration, compared to the results at 0.1 MPa. The optimum NaCl concentration was changed only slightly on changing the hydrostatic pressure. Interestingly, at both 0.1 MPa and 28 MPa there was a dramatic drop-off in growth at NaCl concentrations below 200 mM. The position of this threshold appears to be independent of hydrostatic pressure.

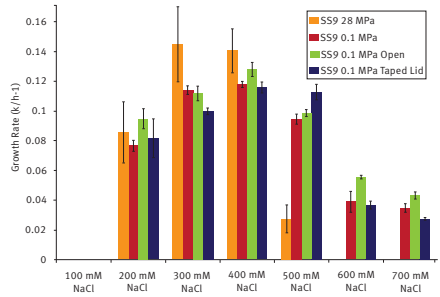

Fig. 2: Measured growth rates, averaged over 33 replicates, for a range of NaCl concentrations, for sealed plates at 0.1 MPa and 28 MPa, for open plates at 0.1 MPa and for plates with taped lids at 0.1 MPa.

These preliminary observations indicate a clear coupling between the response to hydrostatic and osmotic pressure in this bacterium. Comparing the results of our control experiment with the open plate to those for the sealed plate at 0.1 MPa, we conclude that the effects of oxygen availability are minor, although some decrease in growth in the anaerobic plates may be discernable at the highest NaCl concentrations. We also observed good agreement between the results with the taped lid and the sealed plate, at 0.1 MPa, confirming the reliability of our method for measuring growth rates.

Conclusion

In this report, we have demonstrated that the BMG LABTECH microplate reader is well suited for high-throughput assays of microbial growth, even under extreme conditions such as high hydrostatic pressure. We have described preliminary tests of a method for pressure-resistant sealing of 96-well microplates.

Monitoring bacterial cell-to-cell communication "quorum sensing" using a BMG LABTECH microplate reader

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- **Quorum sensing is a phenomenon allowing bacteria to adapt to environmental changes**
- **Effects on quorum sensing were investigated by measuring bioluminescence and bacterial growth**
- **The FLUOstar® OPTIMA was used to measure absorbance and luminescence in parallel**

Introduction

Bacteria can monitor and respond to changes to environmental conditions via a cell density process known as quorum sensing (QS). Bacteria uses this process to monitor their community by producing, detecting and responding to low molecular mass signal molecules, called autoinducers (AI). When the cell density increases the concentration of these signaling molecules will also increase. Once the accumulation of these molecules reaches a threshold, bacteria are collectively able to regulate gene expression and therefore cooperatively regulate their metabolic behavior. Quorum sensing has been shown to regulate a variety of processes in bacteria. These includes bioluminescence and symbiosis in *Vibrio fischeri*, expression of virulence genes in *Pseudomonas aeruginosa*, expression of virulence, surface proteins and biofilm formation in *Escherichia coli*, and biosynthesis of extracellular polymeric substances and pathogenicity in *Erwinia stewartii*. Two major types of quorum sensing molecules (QSMs) have been widely described in literature. The commonest QSM used by Gram-negative bacteria are known as N-acyl homoserine lactones (HSLs) whilst Gram-positive bacteria use amino acids and short peptides (oligopeptide) as their AIs.

Quorum sensing was first described in *V. fischeri*, a Gram-negative bacteria. At low cell density, *Vibrio fischeri* is non-bioluminescent, but when the concentration increases (high cell density), the organism is bioluminescent. The molecular basis for regulation of bioluminescent in *V. fischeri* via quorum sensing has been well studied. The gene cluster responsible for light production consists of eight genes (luxA-E, luxG, luxI, and luxR) (Figure 1).

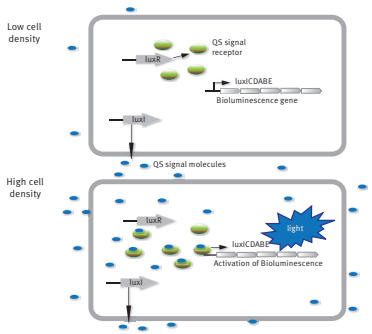

Fig. 1: The HSL-dependent LuxI/LuxR regulatory system of *V. fischeri*.

The regulator proteins responsible for quorum sensing in this organism are proteins encoded by luxI and luxR. LuxI encodes the enzyme AHL synthase, which catalyses the reaction involved in the biosynthesis of HSLs known as N-3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C6-HSL). LuxR encodes the protein which binds to the AI and also activates the luxA-E and luxI operons.

The aim of this study was to elucidate the effect of changes in environmental condition such as growth media as well as the addition of exogenous homoserine lactone on 3-oxo-C6-HSL on the growth and bio-luminescence in *V. fischeri*. A BMG LABTECH micro-plate reader was used to measure absorbance and luminescence in script mode.

Materials & Methods

Bacteria strain and Media

V. fischeri ESR1 and its mutants were kindly supplied by Prof Edward G. Ruby. The ESR1 variants include a signal-negative mutant that does not synthesize the 3-oxo-C6-HSL signal but can still respond to exogenous 3-oxo-C6-HSL, (*V. fischeri* KV240) and a signal-blind strain that produces 3-oxo-C6-HSL but do not respond to 3-oxo-C6-HSL (*V. fischeri* KV267).
All *V. fischeri* strains were grown in Luria-Bertani salt media (LBS), which contains 1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract (wt/vol), 2% NaCl (wt/vol), and 0.3% (vol/vol) glycerol in 50 mM Tris-HCl (pH 7.5) or in seawater tryptone (SWT) which contains 0.5% (wt/vol) tryptone, 0.3% (wt/vol) yeast extract, and 0.3% glycerol (vol/vol) in 70% seawater.

Evaluation of *V. fischeri* growth and bioluminescence under different media and concentrations of homoserine lactone

The growth and bioluminescence of all *V. fischeri* strains were monitored using a multi-mode plate reader from BMG LABTECH. Briefly, overnight cultures of *V. fischeri* strains grown in the above media at 28°C were inoculated to fresh media (1:500 dilution) with or without the addition of exogenous 3-oxo-C6-HSL. 200 µL of diluted cells (quadruplicate) were then transferred into a white 96 well clear bottom microplate (Greiner Bio-One).

The instrument incubation temperature was set at 28°C with continuous shaking at 300 rpm. The OD_{600} and luminescence were then measured every hour for at least 16 h. The *V. fischeri* bioluminescence reading was reported as relative light units (RLU) divided by OD_{600}.

Rev. 08/2009
Keywords: **Bacterial growth, Bioluminescence, Cell density, OD_{600}, Quorum sensing**

Results & Discussion

The increase in cell number over time for *V. fischeri* strains, grown in LBS and SWT can be seen in figure 2. The growth curves represent a typical growth curve under batch conditions with two clearly distinct phases i.e. exponential 2-8 h and onset of stationary phase after 8 h.
There was no significant difference between each strain, suggesting that quorum sensing in *V. fischeri* does not promote or inhibit growth.

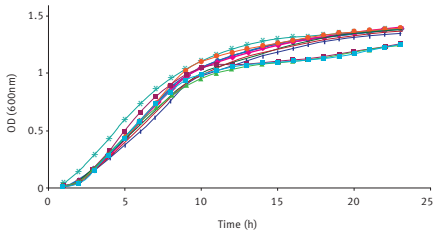

Fig. 2: Batch growth curve of *V. fischeri* strains grown in LBS and SWT with and without addition of 10 µM 3-oxo-C6-HSL.

The expression of bioluminescence by *V. fischeri* ESR1 cultivated in LBS and SWT (with or without 3-oxo-C6-HSL) can be seen in figure 3 and 4 respectively. For *V. fischeri* ESR1, bioluminescence was higher in SWT than in LBS. Earlier activation of bioluminescence was observed when exogenous 3-oxo-C6-HSL was added to *V. fischeri* ESR1 at the onset of growth. For *V. fischeri* KV240, bioluminescence was only observed when exogenous 3-oxo-C6-HSL was added at the onset of growth. This confirms that the strain lacks the ability to produce endogenous 3-oxo-C6-HSL and can only express bioluminescence upon addition of exogenous 3-oxo-C6-HSL. As expected *V. fischeri* KV267, did express bioluminescence with or without the addition of exogenous 3-oxo-C6-HSL since it lacks the ability to respond to the signal molecule.

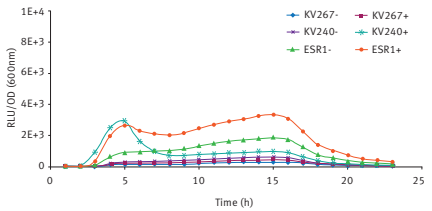

Fig. 3: Bioluminescence expression of *V. fischeri* strains grown in SWT (+ means addition of 10 µM 3-oxo-C6-HSL; - means no 3-oxo-C6-HSL).

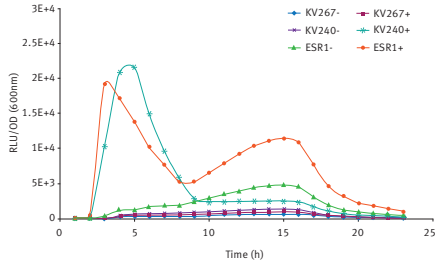

Fig. 4: Bioluminescence expression of *V. fischeri* strains grown in SWT (+ means addition of 10 µM 3-oxo-C6-HSL; - means no 3-oxo-C6-HSL).

Conclusion

In this study, we show that BMG LABTECH microplate readers are a useful tool for understanding quorum sensing in bacteria. The instrument is able to monitor microbial growth and bioluminescence in parallel.

Growth of *Campylobacter* using a microplate reader equipped with ACU

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- **Growth of Microaerophiles using *Campylobacter* as a model**
- **Comparison of growth in a test tube incubator to 96 well plates in an instrument with ACU**
- **Independent Oxygen and Carbon Dioxide regulation allows for optimal growth conditions for different cell types**

Introduction

Campylobacter are Gram negative microaerophilic organisms which are a common commensal of mammals and birds and are one of the most frequently isolated causative agents of human bacterial enteritis worldwide. Infection in humans is frequently associated with the consumption of under cooked poultry and is generally self limiting though it can in a small number of cases result in severe complications such as Guillain-Barre syndrome. Though similar spiral shaped organisms had been observed in stool samples from enteritis patients for many years, *Campylobacter jejuni* was only properly linked with disease in the 1970's when new culture methodologies were developed. *C. jejuni* has quite stringent requirements for growth, namely; a rich nutrient broth, temperatures of 37-42°C, and microaerophilic conditions, i.e. oxygen reduced to 5-8% and carbon dioxide elevated to 10%. It has been suggested that these conditions closely mimic those in the intestine of avian hosts to which the bacteria have become adapted in the wild.

Historically in our laboratory *Campylobacter jejuni* growth studies have been performed in shaking liquid cultures in 15 mL tubes maintained at 42°C with 7% O_2 and 10% CO_2 in a variable atmosphere incubator (VAIN) cabinet; these are then removed temporarily from the incubator to take optical density readings at regular intervals. There are several limitations of this method: the samples are necessarily exposed to laboratory atmospheric conditions and temperature during the time taken for the sampling; the slow doubling time of *C. jejuni* means that ideally samples need to be taken over a 24 hour period; and there is a physical limit to the number of samples and replicates that can be easily handled in the VAIN.

Described here is a comparison of *C. jejuni* growth studies performed using the traditional method of tubes shaking in a VAIN with that of a study completed in a 96 well microplate format using a BMG LABTECH plate reader equipped with the Atmospheric Control Unit (ACU) to independently control oxygen and carbon dioxide.

Materials & Methods

- Clear flat-bottomed 96-well tissue culture plates (Nunc)
- Gas-permeable pre-pierced sealer (FluidX)
- Microplate reader from BMG LABTECH with Atmospheric Control Unit (ACU)

All *Campylobacter* cultures were grown in Mueller Hinton broth (MHB) or on Mueller Hinton agar (MHA) plates (Oxoid). Antibiotics were added at these final concentrations: vancomycin, 10 µg/mL; trimethoprin, 5 µg/mL. Bacterial cultures were grown in sterile Corning 15 mL polypropylene tubes.

VAIN Method:
Strains were recovered from -80°C freezer stocks onto MHA plates containing trimethoprin and vancomycin (VT) and grown overnight in the VAIN (a Whitley VA1000 Workstation; Don Whitley, UK) at 42°C under 10% CO_2 and 7% O_2. Plates with growth were swabbed and re-plated to fresh MHA plates, containing appropriate antibiotics as required, and re-incubated in the VAIN overnight. These plate cultures were used to inoculate 5 mL MHB (+VT) starter cultures which were then incubated with shaking (50 rpm) overnight in the VAIN. The optical density (600 nm) of each starter culture was measured and the cultures were then diluted accordingly in fresh MHB+VT to give a final OD600 of 0.01 (generally a 50- to 80-fold dilution).

Triplicate cultures of 8 mL in 15 mL tubes were prepared for each strain and placed in the VAIN shaking at 50 rpm. Every few hours the cultures were removed from the VAIN, a small sample was taken, and the optical density at 600 nm was determined using cuvettes and a spectrophotometer.

Using BMG LABTECH microplate reader with Atmospheric Control Unit:
Starter cultures were generated in the VAIN using the same method as above and the strains were diluted in fresh MHB+VT to give cultures with OD600 of 0.01. Multiple 200 µL aliquots of diluted cultures were inoculated into individual wells of a 96 well microplate – for these experiments we used 6 technical replicates and two MHB blanks per strain. The plate was then sealed with a gas permeable pre-pierced seal taking care to position the pierced slits away from the centre of the wells to avoid interference with the absorbance measurements.
The plate was then incubated in the microplate reader at 42°C with the Atmospheric Control Unit set to independently control the O_2 to 7% and the CO_2 to 10%. Oxygen Free Nitrogen and Carbon Dioxide bottles were connected at 30psi. Absorbance at 600 nm was measured (20 flashes/cycle) for forty-nine cycles of 30 minutes with continual shaking (double orbital) at 500 rpm between measurements.

Rev. 08/2011
Keywords: **ACU Atmospheric Control Unit, *Campylobacter*, CO_2, Microbial growth, OD_{600}**

Results & Discussion

Typical growth curves for *Campylobacter* grown in tubes in the VAIN are shown in Figure 1.

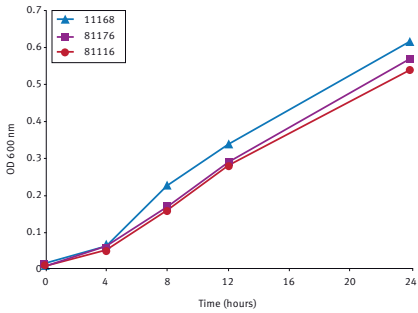


Fig. 1: Growth of *C. jejuni* genome strains over a 24 hour period grown in shaking tubes in a VAIN.

Growth typically exhibits a 4-5 hour lag phase before growing exponentially to an OD600 maximum of 0.5-0.6 depending upon the strain. Growth for *Campylobacter* strains in 96 well plate format incubated in the microplate reader with Atmospheric Control Unit is shown in Figure 2.

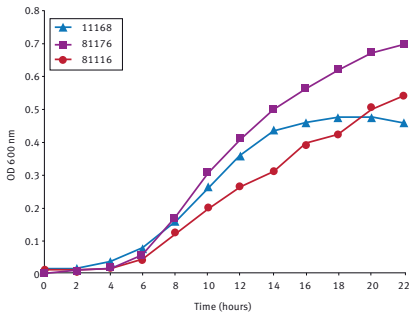


Fig. 2: Growth of Campylobacter isolates over a 24 hour period in the FLUOstar® Omega.

The lag phase exhibited during growth in the microplate format appears to be slightly longer than in the tubes however the growth curves are very similar and the maximum growth levels achieved after 24 hours are comparable.
The programmed automated data collection provides the ability to measure growth curves over extended periods with readings at regular intervals. This generates growth curves with much more detail which is important when comparing subtleties in response.

The capacity to control both gases independently provides the ability to test growth under different conditions quickly and simply. Furthermore different O_2 and CO_2 concentrations can be set for other micro-organisms, *Salmonella, Neisseria* etc, without the need to change the gases or to connect bespoke gas mixes.
The ability to perform and monitor growth simply in a multi-parallel manner provides us with the capacity to rapidly perform growth studies analysing multiple mutants across a range of *campylobacter* strains. Additionally the added potential of being able to work under different oxidative conditions will allow us to undertake comparative growth experiments which we would not have previously considered due to the constraints of having multiple users in one VAIN cabinet.

Conclusion

Comparing growth of *Campylobacter* in tubes incubated in a VAIN incubator to those grown in 96 well plates in the microplate reader with atmospheric control clearly demonstrates that the growth of this fastidious organism can be transferred to a 96 well format. This is made easy by the design of the BMG LABTECH instrument providing temperature control and shaking together with the Atmospheric Control Unit that facilitates the independent control of both O_2 and CO_2 in the reader.

Atmospheric Control Unit (ACU)

CLARIOstar®

Omega Series

A new way to test the free amino nitrogen content in alcoholic beverages with the SPECTROstar® *Nano*

Gary Spedding[1], Nathan R. Harrison[1], Franka Ganske[2] and EJ Dell[2]
[1]Brewing and Distilling Analytical Services, LLC [2]BMG LABTECH

- **Ninhydrin-based assay detects free amino nitrogen (FAN) in beverage and food analysis**
- **Scaling down the original assay reduces reagents and limits waste**
- **SPECTROstar® *Nano* is the optimal reader to measure absorbance in cuvettes and microplates**

Introduction

Standard Brewing quality control analyses include the test for free amino nitrogen (FAN). This allows an estimation of the protein-content and is a beer quality indicator. The traditional way to determine the protein content of beers is based on Kjeldahl assays which are elaborate and quite expensive. An alternative to this assay is the ninhydrin-based FAN assay for cuvettes. A standard assay used for 40-50 years by the brewing industry. Primary amino acids can be detected with the help of ninhydrin. The result is a blue dye that shows an absorbance maximum at about 570 nm (Fig. 1). The amino acid glycine is used as a standard substance for this assay.

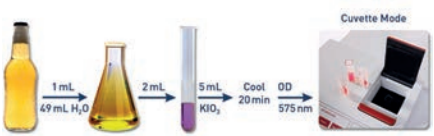
The next step was to reduce the volume for the standard cuvette assay and to move to a multi-well format. This was pioneered by Starcher and staff and our lab, and it was published in the brewing literature. Scaling down the test reduces the demand for expensive reagents, it limits waste and thus makes for simpler and less expensive disposal.

The SPECTROstar *Nano* from BMG LABTECH combines cuvette and microplate measurements in one instrument. The integrated spectrometer can take spectra over time and the MARS data evaluation software shows overlay plots of all measurements.

Materials & Methods

- Clear 96-well plates from Greiner
- SPECTROstar *Nano*, BMG LABTECH

Standard FAN assay at pH 6.8:



Ninhydrin Color Reagent: 4 g anhydrous Na_2HPO_4, 6 g KH_2PO_4, 0.5 g ninhydrin and 0.3 g fructose are dissolved in a total of 100 mL distilled water. Stock may be stored refrigerated up to 2 weeks in an amber bottle.

Glycine Standard Stock: Exactly 107.2 mg glycine is dissolved in distilled water and brought to exactly 100 mL. Standard stock may be stored at 0 - 4°C to avoid growth of molds.

Glycine Standard Solution: 1 mL glycine standard stock is diluted into 100 mL (final vol.) with distilled water. The standard contains 2 mg amino-nitrogen/L. Use freshly made for each daily assay run.

Dilution solution: 2 g of potassium iodate (KIO_3) is dissolved in 600 mL distilled water and 400 mL 96% ethanol added. Stable long-term at 4°C; use close to room temperature to prevent condensation on cuvettes.

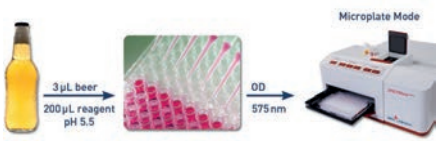
Assay: 1 mL of beer is diluted to 50 mL with distilled water and 2 mL transferred to 16 X 150 mm test tubes. Ninhydrin color reagent (1 mL) is added and the loosely covered tubes heated in a boiling water bath for 16 min. The tubes are transferred to a cold water bath to bring to 20°C (within 20 minutes) and then 5 mL of dilution reagent is added, mixed and the absorbance immediately recorded at 575 nm against a blank containing 2mL of water in place of the sample.

Microplate FAN assay at pH 5.5

Ninhydrin Stock Solution: 8 g of ninhydrin is dissolved in 300 mL of ethylene glycol and 100 mL of 4 N sodium acetate pH 5.5 buffer* is then added (*544 gm. sodium acetate tetrahydrate + 400 mL glacial acetic acid in 1L water). Stock is stable at room temperature for at least 6 months.

Stannous Chloride Solution: 500 mg $SnCl_2$ is dissolved in 5 mL ethylene glycol. Solution is stable at room temperature for at least 6 months.

Ninhydrin Reagent/Working Solution**: Prior to the assay, 25 µL of $SnCl_2$ solution is added to every 1 mL of ninhydrin stock solution and mixed well.



Assay: 2 µL (0.4 µg N) of glycine standard or 2 µL of beer or grape juice is added to a 96 well micro-well plate and 100 µL of the pH 5.5 acetate buffered ninhydrin reagent is then added. The plate is heated for 10 min at 104°C. The absorbance is recorded simultaneously for multiple samples at 575 nm in the SPECTROStar *Nano* microplate reader.

Reduced volume cuvette FAN assay at pH 5.5

Assay: Beer or grape juice (30 µL) or 20 µL (4 µg N) glycine standard is added to 200 µl of the pH 5.5 acetate buffered ninhydrin reagent* (see microplate assay above) and placed in a boiling water bath for 10 min. After 10 min samples are removed and 2.8 mL cold water added, the tubes are then vortexed and the absorbance at 575 nm recorded in the SPECTROstar Nano against a blank containing 30 µL of water in place of the sample.

Rev. 05/2012
Keywords: **Beer, Beverages, Cuvette, Food, Spectral analysis**

Controls and calculations

Tests should be in duplicate or triplicate for each sample, water blanks, and glycine standard. The average blank value is subtracted from each sample and the standard glycine. Then the blank corrected sample net (average) absorbance is divided by the net absorbance of the standard glycine and multiplied by 2 and the dilution factor (50 for beer diluted 1 mL into 50 mL or 100 for beer wort diluted 1 mL into 100 mL) to obtain FAN content in ppm. [Samples, including mash samples, should be free of haze/ debris before dilution – filter or centrifuge.]

Results & Discussion

Wavelength Optimization

The optimal wavelength to measure this assay can be obtained by the scanning capabilities of the spectrometer in the SPECTROstar *Nano*. Spectra were taken from the ninhydrin reaction either with glycine or a beer sample (Fig. 1).

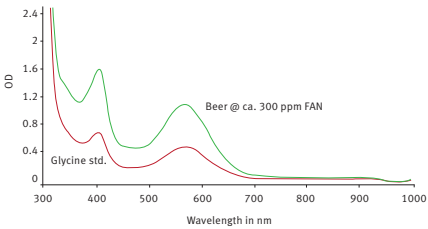


Fig. 1: Spectra captured for a glycine standard (red line) and a beer sample (green line).

The spectra taken with the SPECTROstar *Nano* proved that there is a clear absorbance maximum at about 570-575 nm.

Reaction times required for completion of the FAN assays

The reaction times of 4 different ninhydrin-based FAN assays were compared. Next to the already explained three assays (standard FAN pH 6.8, microplate FAN pH 5.5 and reduced volume rFAN cuvette pH 5.5, please see Materials and Methods) a second reduced cuvette FAN assay at pH of 6.8 was investigated. The result is shown in Fig. 2.

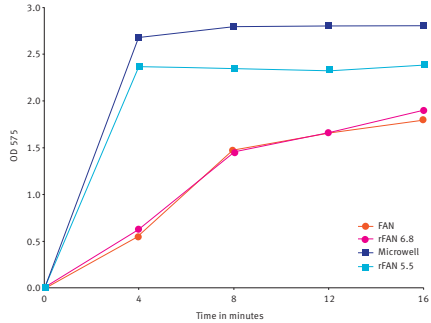


Fig. 2: Reaction times required for completion of 4 different FAN assays).

It turned out that the reaction rates are different for the assays. It is seen that the standard FAN and rFAN pH 6.8 reactions required at least 16 minutes to approach, at best, maximum absorbance. The rFAN pH 5.5 and the microplate FAN assay attaining maximal values and stable plateaus within 8 minutes. There was no loss of absorbance in any of the assays when incubated up to 20 minutes.

Conclusion

We showed that the SPECTROstar *Nano* was very well suited to measure the FAN microplate and cuvette assays for beer samples. The new reduced volume cuvette and microplate based FAN deal with a more concentrated ninhydrin stock solution, no extended cooling time is needed and no dilution/quench solution is required. There is further no need to run dilutions on beer/wort samples prior to sampling. These are all advantages compared to the 40-50 years old standard FAN cuvette assay.

The assays were optimized further in terms of pH and buffers and should prove useful for many brewing, enology, distilling and food laboratories for the routine determination of free or total useable nitrogen.

Biocolor's APOPercentage Apoptosis Assay™ on BMG LABTECH's microplate reader

Morrow, J.C. and Steele D.
Biocolor Ltd. 8, Meadowbank Road, Carrickfergus, Co. Antrim, N. Ireland, BT38 8YF

- **Monitors occurrence of apoptosis in live, mammalian, anchorage-dependent cells**
- **Detects and quantifies percentage of apoptosis in cell population**

Introduction

Apoptosis is a multistage process during which activity of caspase enzymes fluctuates, DNA becomes fragmented and phosphatidyl serine is transferred to the outside of the cell membrane.

Common methods for analysis include:

1. Caspase activity assay, either colorimetric, fluorescent, luminescent or antibody based.
2. TUNEL assay, based on DNA fragmentation.
3. Annexin-V assay, based on the binding of dye or fluorescently conjugated Annexin-V to phosphatidyl serine which has translocated to the cell membrane exterior during apoptosis.

Necrotic cells must be distinguished using propidium iodide.

APOPercentage Principle

Transfer and exposure of phosphatidyl serine to the exterior surface of the cell membrane has been linked to the onset of apoptosis. Phosphatidyl serine transmembrane movement results in the uptake of APOPercentage Dye by apoptotic committed cells. Dye uptake continues until blebbing of the apoptotic committed cell occurs. No further dye can then enter the defunct cell and the dye that has accumulated within the cell is not released. Necrotic cells do not retain the dye. Dyed cells can be counted using a light microscope or the dye can be released from the cells and measured spectrophotometrically as in the method below.

Materials & Methods

- Minimum cell culture facilities
- BMG LABTECH microplate reader
- 24 and 96 well microplates

Protocol

1. Seed a 24-well tissue culture plate with 5 x 10^4 cells/well in 500 µL culture medium and incubate the cells at 37°C/5% CO_2, until confluence is reached (~ 24 h).
2. Prepare dilutions of test apoptotic agent(s) at selected concentrations using the suggested layout below. Controls (-ve & +ve) should be included with each experiment.

Reag. Blank	Reag. Blank	-ve Control	-ve Control	+ve Control	+ve Control
Sample 1	Sample 1	Sample 4	Sample 4	Sample 7	Sample 7
Sample 2	Sample 2	Sample 5	Sample 5	Sample 8	Sample 8
Sample 3	Sample 3	Sample 6	Sample 6	Sample 9	Sample 9

3. Make up double quantity of Reagent A. Use half the volume to prepare Reagent B.

	Reagent A (500µL/well)	Reagent B (500µL/well)
Reagent Blank	Culture medium / serum	Reagent A
Negative Control (0% apoptosis)	Culture medium / serum	Reagent A + 5% v/v dye
Positive Control (100% apoptosis)	Culture medium / serum + reference apoptotic agent	Reagent A + 5% v/v dye
Test Samples (>0%, <100%)	Culture medium / serum + test apoptotic agent	Reagent A + 5% v/v dye

4. Remove the culture medium from each well of the incubated plate and add 500 µL of Reagent A, supplemented v/v with serum (if required), to all wells.
5. Incubation time for apoptotic inducer/inhibitor will depend on apoptotic agent used. 30 min before this time period is reached remove Reagent A. Immediately replace with 500 µL Reagent B and incubate for the remaining 30 min, at 37°C/5% CO_2.
6. Remove Reagent B from each well using a pipette. Gently wash the cells twice with 1000 µL/well PBS to remove non-cell bound dye. (NOTE: Careful aspiration is advised as some apoptotic agents can cause detachment and loss of cells).
7. Add trypsin (50 µL) to each well and incubate for 10 minutes at 37°C/5% CO_2. Tap the plate gently by hand after 5 minutes and again after 10 minutes to detach cells from the plastic, cell culture treated surface.
8. Now add 200 µL Dye Release Reagent to each well and shake plate for 10 minutes.
9. Transfer contents of each well (250 µL) to a 96 well flat bottom plate and read absorbance at 550 nm. Bubbles in wells affect results, burst with clean pin or transfer 200 µL instead of 250 µL to microplate.

This experiment was carried out with triplicate samples.

Rev. 09/2012
Keywords: **Apoptosis, Biocolor, Caspase, H_2O_2, Membrane permeability**

Instrument Settings

Measurement Type:	Absorbance
No. of Flashes per well:	22
Excitation:	550 nm
Shaking Frequency (rpm):	300
Shaking Mode:	double orbital
Additional Shaking Time:	3s before plate reading
Positioning Delay (s):	0.2

Results & Discussion

1. Follow protocol as above to obtain absorption data.
2. Subtract the mean value of reagent blank replicates from test results.
3. Plot mean absorbance values ± standard error of the mean in a bar chart (Fig. 1) or as a percentage of the Positive Control absorbance value.

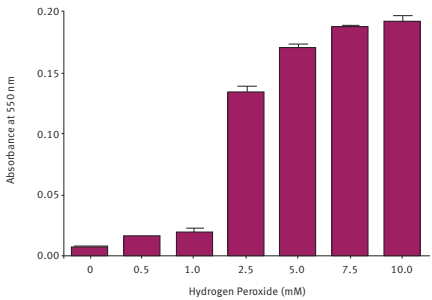


Fig. 1: Colorimetric Quantification. Graph Showing Effect of Hydrogen Peroxide (0 – 10mM) on CHO Cells. Results expressed as mean absorbance for triplicate wells ± S.E.M. (n = 3). Exposure time to H_2O_2 was 4 hours.

Conclusion

The microplate reader from BMG LABTECH provide a fast, accurate and consistent method for quantifying apoptosis colorimetrically.

Background

Biocolor is a UK company based in Carrickfergus, N. Ireland, with a network of distributors throughout the world. Biocolor's expertise lies in its unique range of extracellular matrix assays for use with mammalian cells, tissues and fluids: Sircol™- Collagen, Blyscan™- Glycosaminoglycan and Fastin™-Elastin. VolCol is Biocolor's range of bovine and rat collagen products.

More information can be found at www.biocolor.co.uk

Lonza's kinetic kit for endotoxin detection using BMG LABTECH's microplate reader and MARS data analysis

Chris Quinlan and Carl Peters,
BMG LABTECH

- **To avoid unwanted inflammatory responses it is mportant that DNA samples are endotoxin free**
- **The Lonza endotoxin quantitation kit was performed on a filter-based microplate reader**
- **Use of MARS Data Analysis simplifies data interpretation**

Introduction

Endotoxins or lipopolysaccharides (LPS) are undesirable byproducts of gram-negative bacterial preparations which are often found in plasmid DNA and ovalbumin preps. LPS is located in the outer membrane of the bacteria. Even trace amounts can cause a significant inflammatory response. The presence of endotoxin in the blood is called endotoxemia and can lead to sepsis in mammals. Therefore, endotoxins must be detected and eliminated from DNA and protein preparations to avoid unwanted responses in both in vivo and in vitro assays.

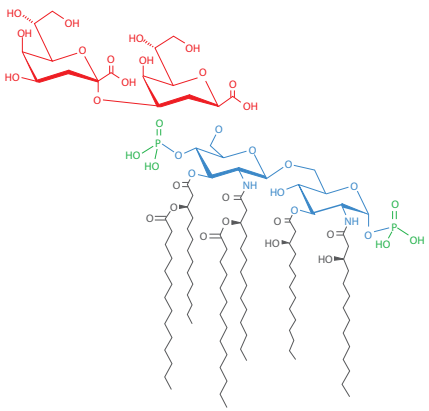
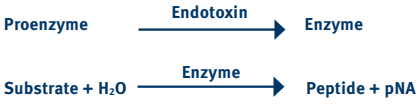


Fig. 1: Structure of the (3-deoxy-D-manno-octulosonic acid)2 Lipid A endotoxin from E. coli K-12. This fi gure is licensed under the Creative Commons Attribution-Share Alike 3.0 Unported licenses and was adapted from: http://www.jlr.org/content/47/5/1097.full.

Lonza has developed a kinetic endotoxin quantitation kit, which utilizes the coagulation properties of horseshoe crab blood in the presence of even low levels of endotoxin. The clottable protein has been isolated and is the active component of the Limulus Amebocyte Lysate (LAL). In the presence of endotoxin, a proenzyme in the LAL is activated which in turn cleaves a colorless peptide, Ac-Ile-Glu-Ala-Arg-pNA, resulting in the release of p-nitroaniline (pNA) which can be detected by continuous absorbance measurements at 405 nm.

Assay Principle



The concentration of the endotoxin is calculated by comparing the reaction times of samples to solutions containing known amounts of endotoxin.

The reaction time is typically defined as the time it takes to produce a 0.2 OD change in absorbance at 405 nm. The endotoxin concentration is inversely proportional to the reaction time so a smaller reaction time indicates a higher endotoxin concentration. The microplate reader offers an easy way of measuring the Kinetic Endotoxin assay and in conjunction with MARS data analysis software can produce reaction times. MARS can also plot the standards and interpolate unknown samples from a linear regression or polynomial fit.

Materials & Methods

- Corning 96 well Microplate, Clear
- Filter-based or spectrometer equipped microplate reader from BMG LABTECH
- Lonza Kinetic-QCL Endotoxin Kit

100 µl of standards and unknowns were measured for absorption at 405 nm in duplicate for a total of 100 min in plate mode (slow kinetics). A reading was taken every 2.5 min for a total of 40 points. Standards included 0.005, 0.05, 0.5 and 5.0 EU/ml, where EU = endotoxic units, a comparative measure of endotoxin activity. Since a baseline correction will be applied, blanks are not required for optimal assay performance.

Instrument settings

Detection Mode:	Absorbance, plate mode kinetic
Optics:	405 nm
No. of cycles:	40
Cycle Time:	150 sec

Rev. 07/2013
Keywords: **Endotoxin, Horseshoe crab, LAL assay, Limulus amebocyte, Lipopolysaccharide**

Results & Discussion

The absorbance measurements over time resulted in different signal curves (Fig. 2).

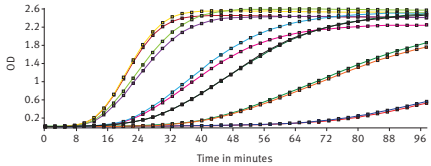


Fig. 2: Signal curves for several endotoxin samples. Figure is directly taken from the MARS data analysis software.

The kinetic data was evaluated utilizing the MARS data analysis software from BMG LABTECH. A baseline correction was applied to the raw data by subtracting cycle 1. The reaction time was calculated by performing a "time to threshold calculation" on the baseline corrected raw data. The threshold value was set for 0.2 OD producing the resulting reaction time in seconds. The average reaction time was plotted against concentration in EU/ml using a linear regression model (Fig. 3).

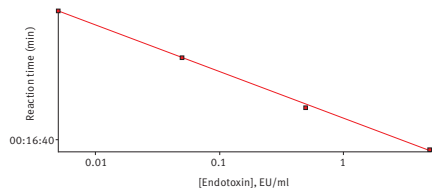


Fig. 3: Linear Regression Fit of Standards (log/log).

The resulting standard curve fit had an R2 value of 0.999 allowing for reliable interpolation of unknown samples (Table 1).

Sample	[Endotoxin], EU/ml calculated from Linear Regression Fit
Unknown 1	4.59
Unknown 2	0.38

Alternatively, the MARS data analysis software facilitated the plotting of standards utilizing a polynomial curve fit. It is recommended that the polynomial order be one less than the number of standards used. In this case a 3rd order polynomial fit could be used.

The MARS data analysis software further allows for the creation of a template for these and other sophisticated calculations (Fig. 4) to provide immediate data reduction and curve fits as soon as the assay is completed.

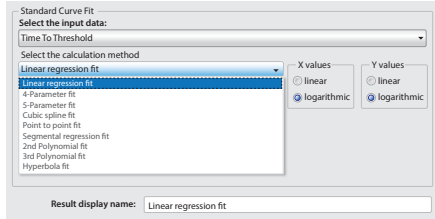
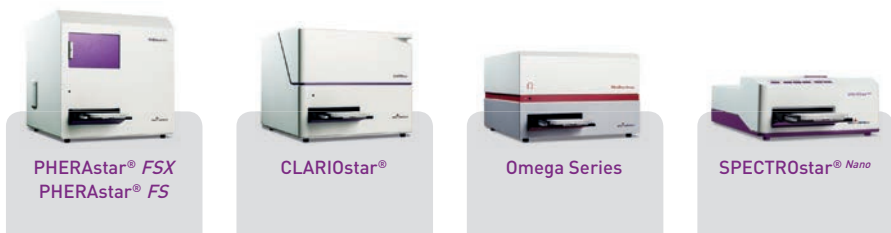


Fig. 4: Overview of standard curve fits in the MARS data analysis software.

Conclusion

The combination of the sensitive microplate reader and the powerful MARS data analysis package allows for easy handling of complex assays such as the Kinetic-QCL Endotoxin kit from Lonza. The MARS data analysis package is standard with all BMG LABTECH readers and can be installed on other computers in the same lab at no additional charge. This assay kit can also be measured by all BMG LABTECH microplate readers that can measure absorbance including the Spectrostar Nano, PHERAstar® *FS*, FLUOstar® Omega, POLARstar® Omega, and CLARIOstar®.



Low-volume protein measurements (280nm): validating the LVis Plate over many concentrations

Sarfaraj Topia, Alison Turner
UCB Pharma, Bath Road, Slough

- **Different instrumentation was used to measure UV protein absorbance at 280 nm**
- **Low and high range protein concentrations were successfully measured in 2 µl with the LVis plate on a microplate reader from BMG LABTECH**

Introduction

Measurement at the working concentration of an experiment in a high throughput manner is useful especially when dilution of the protein may affect the solubility characteristics. This would also be advantageous where accurate protein concentration is required on numerous samples (for example, column elution fractions), where dilution would be time consuming and potentially introduce pipetting errors. This coupled with the ability of being able to analyse the samples at low volume would also offer an added advantage when quantities of sample are limited.

The LVis Plate is a proprietary absorbance microplate from BMG LABTECH and is perfectly suited for low volume protein quantitation. It consists of sixteen microdrop well sites for 2 µL samples and adheres to the 96-well microplate format definition. The microdrop well sites are also easily accessible to wipe clean for further measurements.

The aim was to demonstrate that the LVis Plate was able to measure protein concentration at two different concentration ranges (0.2 to 18 mg/mL) reliably as compared to alternative methods, a UV/Vis spectrophotometer (manual cuvette sampling), and an alternative high throughput technique (NanoDrop).
The LVis Plate was used with a microplate reader (BMG LABTECH), which has an ultra-fast UV/Vis spectrometer. This technology captures an entire spectrum (220 - 1000 nm) in <1 sec/well. The LVis Plate is compatible with the SPECTROstar® *Nano*, Omega series, PHERAstar® *FS*, and CLARIOstar® microplate readers from BMG LABTECH.

Materials & Methods

- Sample Protein Stock Solution 30 mg/mL in 20 mM sodium acetate, 100 mM sodium chloride, pH 5.0
- Working buffer: 50 mM sodium acetate, 100 mM sodium chloride, pH 5.0 [filtered (0.22µm)]
- LVis Plate (BMG LABTECH)
- Cary Bio 50, UV/Vis Spectrophotometer
- NanoDrop, Thermo Scientific, UK

Experiment 1
To compare the reproducibility at a low concentration range (up to 1 mg/mL). The sample protein is a full length monoclonal antibody.
Serial dilutions of the protein stock solution were made ranging from 0.2 mg/mL to 1.0 mg/mL in buffer (detailed above). The absorbance at 280 nm was measured using the LVis plate (BMG LABTECH) and compared with results obtained from alternative spectrophotometers, a UV/Vis Spectrophotometer and the NanoDrop. Measurements were made in triplicate for each method.
Aliquots of 2 µL were used for the LVis Plate and the NanoDrop, whereas 100 µL was used for the UV/Vis spectrophotometer. In each case the samples were blanked against the buffer (detailed above).
The mean and the standard deviation of the absorbance readings at 280 nm at each concentration for each method was derived and then plotted against nominal concentration (Figures 1 - 4).

Experiment 2
To compare reproducibility at a higher concentration range (up to 18 mg/mL).
This experiment only compared the LVis Plate and NanoDrop since the linearity of the UV/Vis spectrophotometer is known to be unreliable at the higher concentration range and hence would not provide a fair test. Dilutions of the protein stock were made from 18 mg/mL to 1 mg/mL in buffer (detailed above). Triplicate measurements were made and the data plotted as in Experiment 1 (Figure 5).

Results & Discussion

Experiment 1
At concentrations ranging from 0.2 mg/mL and 1.0 mg/mL, the mean absorbance at 280nm (OD) was plotted against the concentration and the linear regression fit was calculated.
A high R squared value (R^2) in excess of 0.99 was obtained for the UV/Vis Spectrophotometer (Figure 1), LVis Plate (Figure 2) and the NanoDrop (Figure 3) .

An overlay of the data obtained from each measurement technique (Figure 4) showed that there was good correlation between the three methods.

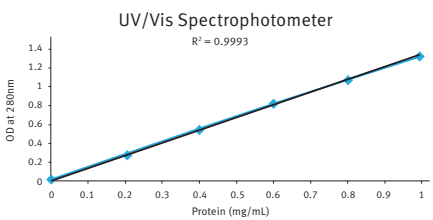


Fig. 1: Results obtained through Cary 50 Bio Spectrophotometer.

Rev. 07/2013
Keywords: **A280, Low-volume, LVis Plate, Protein, Spectrometer, UV absorbance**

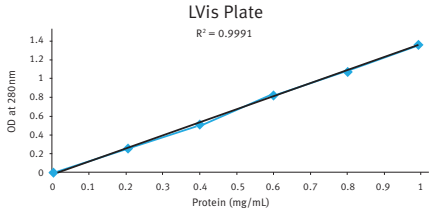


Fig. 2: Result obtained from LVis Plate from BMG LABTECH, UK.

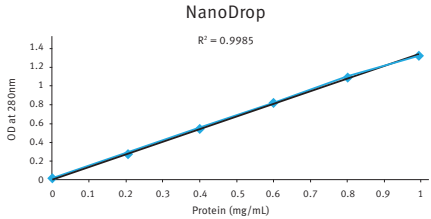


Fig. 3: Results obtained from NanoDrop from Thermo Scientific, UK.

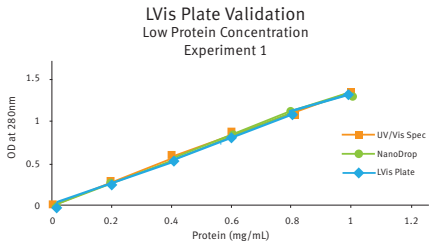


Fig. 4: Overlapped profiles of all three measurements.

Experiment 2

This experiment was performed to check the reliability of the LVis Plate to measure the absorbance at higher concentrations (up to 18 mg/mL) compared with the Nano Drop only. The mean absorbance values at different concentrations were plotted against the back-calculated concentration to show linearity.

The R squared values (R^2) were in excess of 0.99 for both the LVis Plate and NanoDrop and good correlation was observed between the two methods as illustrated by the overlaid results (Figure 5).

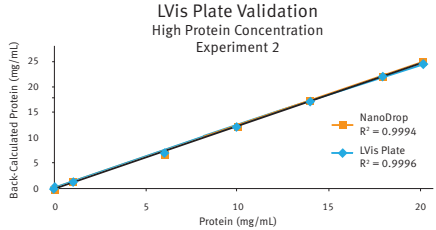


Fig. 5: Overlapped profiles of results obtained from LVis Plate and NanoDrop.

Conclusion

All three instruments for the measurement of concentrations up to 1 mg/mL showed equivalent results. At concentrations up to 18 mg/mL, the LVis Plate and the Nano Drop (an alternative high throughput method) showed comparable results.

Overall, the LVis Plate proved to be a reliable, low volume high throughput method for measurement of protein concentrations from 0.2 mg/mL to 18 mg/mL. This was validated against existing alternative spectrophotometric techniques.

Identification of novel haemoglobin-modifying activity in snake venom libraries

Paul Hayter[1], Anthony Baines[2], Lorraine Croucher[3], Catherine Wark[3] and Steven Trim[1]
[1]Venomtech Ltd [2]University of Kent [3]BMG LABTECH

- **Snake venoms have wide-ranging effects on haemostasis**
- **BMG LABTECH microplate reader used to screen Venomtech's T-VDACV snake venom array**
- **Novel haemoglobin-modifying activity identified**

Introduction

Venoms, particularly those from snakes, have long been known to affect haemostasis in prey species and human victims. Previously discovered venom activities include; thrombosis, haemolysis, hypotension, oedema and haemorrhage. However, knowledge of the direct effects of venoms on mammalian haemoglobin are very rare in the literature and restricted to saliva from haematophagic animals. To further explore the nature of venom activity, the authors set about screening a Cardiovascular – Venom Discovery Array (T-VDA^{CV}) for effects on haemostasis, using a simple 384 well assay employing spectrometer-based microplate reader.

Materials & Methods

- Sheep blood (Harlan laboratories)
- Rabbit blood (University of Kent)
- Clear 384 well plates (Greiner)
- Cardiovascular – Venom Discovery Array (T-VDA^{CV})
- Spectrometer-based microplate reader (BMG LABTECH)

Sheep blood or rabbit blood were lysed by addition of two volumes of distilled water and clarified by centrifugation. Crude snake venoms (30 mg/ml) were derived from the Venomtech T-VDA^{CV} array.

Table 1: Snake venom was taken from the following snakes.

Viperidae family	Short form	Elapidae family	Short form
Bitis gabonica rhinoceros	B.grh	*Naja nigricollis*	N.nig
Agkistrodon contortrix contortrix	A.cco	*Aspidelaps lubricus*	A.llu
Atheris squamigera	A.squ	*Dendroaspis polylepis*	D.pol
Crotalus basiliscus	C.bas	*Pseudechis rossignoli*	P.wei
Cryptelytrops purpureomaculatus	T.pur		

Crude venom, either neat or diluted in phosphate-buffered saline (PBS), was mixed with an equal volume of lysed rabbit blood (neat) or lysed sheep blood (diluted 1:2 in PBS) giving a final assay volume of 20 µl. Sheep blood was diluted to produce comparable absorbance readings to that of the rabbit. Plates were incubated at 30°C in the microplate reader and the absorbance spectrum (400-700 nm) read at 30 minute intervals over 16 hours.

Results & Discussion

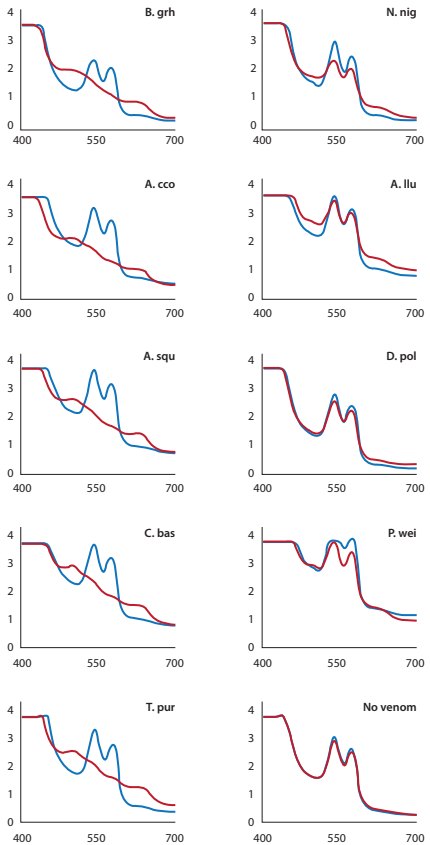


Fig. 1: **Screening a snake venom library for haemoglobin-modifying activity** Haemoglobin absorbance spectrum before (blue trace) and after 16 hours' incubation (brown trace) with venom from members of the Viperidae family (left column), the Elapidae family (right column) or in the absence of venom (bottom right). The x-axis shows wavelengths in nm, the y-axis exhibits the corresponding OD values.

In the absence of snake venom, lysed rabbit blood exhibits absorbance peaks at 540 nm and 570 nm, characteristic of oxyhaemoglobin (HbO_2) and the spectrum remained unchanged after 16 hours' incubation at 30°C (Figure 1).

When incubated with certain snake venoms, particularly those from the Viperidae family, there was a distinct shift in the absorbance profile with a reduction of

Rev. 05/2014
Keywords: **Absorbance profile, Haemoglobin/haemostasis, Oxyhaemoglobin HbO_2, Snake venom, Spectral analysis**

HbO_2 absorbance peaks and the appearance of peaks at 500 nm and 630 nm, a profile characteristic of oxidised haemoglobin (methaemoglobin). Members of the elapidae family generally showed little activity in this assay but there was a detectable shift in the absorbance profile in the presence of the venom of the black-necked spitting cobra, *Naja nigricollis*. Further analysis of *N. nigricollis* venom, using lysed sheep blood (diluted 1:2 in PBS), demonstrated a clear time-dependent shift in the absorbance profile from that typical of HbO_2 to one more characteristic of methaemoglobin (Figure 2).

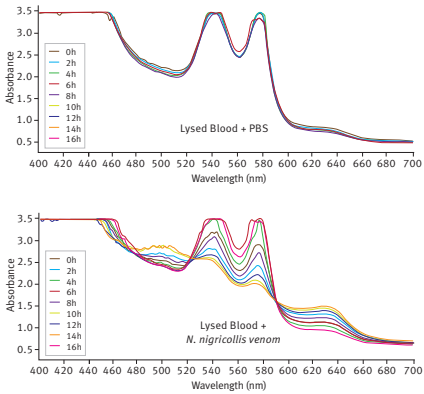


Fig. 2: T**ime-dependent change in haemoglobin absorbance spectrum in the presence of *N.nigricollis* venom** Lysed sheep blood incubated with PBS only (upper graph) or *N. nigricollis* venom (lower graph). Spectrum recorded at 2 hour intervals.

Gel-based assays showed that this spectral shift was not due to haemoglobin proteolysis (data not shown). It is also notable that oxidation of haemoglobin in the presence of *N. nigricollis* venom was not prevented by addition of reducing agents (dithiothreitol at 1 mM or 5 mM) whereas heat-treatment of venom (65°C or 95°C) resulted in loss of activity in this assay (data not shown.) Taken together, these data suggest that the oxidation of haemoglobin, may be enzyme-mediated and represents novel haemoglobin-modifying activity in Viperid and Elapid snake venom.

Conclusion

A simple assay employing spectral analysis of haemoglobin reveals novel haemoglobin-modifying activity in snake venom and while this effect was most pronounced in the venoms from the viperidae family, at least one elapid snake, *N. nigricollis* also exhibited this activity.

The microplate reader from BMG LABTECH has facilitated the design of a simple 384 well assay revealing (through wavelength scanning) novel activities in a snake venom library.

UV absorbance DNA quantitation

Franka Ganske
BMG LABTECH

- **High degree of linearity from 0.1 to 100 µg/mL DNA**
- **MARS evaluation software offers integrated extinction coefficients for dsDNA, ssDNA and RNA**
- **Measure a full absorbance spectrum in less than 1 second per well**

Introduction

One of the most common methods for nucleic acid detection is the measurement of solution absorbance at 260 nm (A260) due to the fact that nucleic acids have an absorption maximum at this UV wavelength. Although a relatively simple and time-honored method, A260 suffers from low sensitivity and interference from nucleotides and single-stranded nucleic acids. Furthermore, compounds commonly used in the preparation of nucleic acids absorb at 260 nm leading to abnormally high quantitation levels. However, these interference and preparation compounds also absorb at 280 nm leading to the calculation of DNA purity by performing ratio absorbance measurements at A260/ A280.

$$\frac{A260}{A280} = 1.7 \text{ to } 2.0 \text{ for "pure" DNA}$$

Therefore, constructing an absorbance ratio between these two absorbance wavelengths can provide an estimate of sample purity. As a general rule any preparations with an A260/A280 greater than approximately 1.7 is called "pure".

Comparing Results of a *Spectrophotometer and a Microplate Reader*

The absorbance measurement is governed by Beer's Law.

$$A = \varepsilon bc$$

Where A is absorbance, ε is the molar extinction coefficient, b is the path length, and c is the analyte concentration. When the molar coefficient and path length are constant, absorbance is proportional to the concentration.

For a standard cuvette reader, the path length is usually defined as 1 centimeter. Therefore, with a conventional absorbance reading an A260 of 1.0 OD corresponds to 50 µg/ml dsDNA solution. In a microplate reader, the same DNA concentration measured will lead to a smaller OD value (about 0.7 OD) because of the smaller path length in a microplate well. The integrated spectrometer in the BMG instrumentation offers a path length correction feature that allows fast determination of DNA concentration in samples as well as results comparable to cuvette-based measurements.

Alternative methods to quantitate DNA than absorbance are fluorescent techniques that are much more sensitive and specific for DNA. The Quant-iT PicoGreen® dsDNA Quantitation Reagent from Life Technologies® for example is a highly sensitive fluorescent assay for double stranded DNA (dsDNA) detection.

Materials & Methods

All materials were obtained through normal distribution channels from the manufacturers stated.

- UV-Star plates, 96-well, Greiner Bio-One
- Deoxyribonucleic acid, Activated from calf thymus, lyophilized powder, Sigma-Aldrich
- Distilled water
- spectrometer-based BMG LABTECH microplate reader

In addition, consumables such as pipette tips and microcentrifuge tubes were used as needed from various manufacturers. The DNA from calf thymus was solved in distilled water to a final concentration of 1 mg/mL. From this stock solution further dilutions were performed yielding different DNA standards ranging from 0.1 to 100 µg/mL. Four replicates of 350 µL aliquots of each standard were pipetted into the 96-well UV plate. Additionally, 16 replicates of 350 µL aliquots of distilled water were pipetted into the plate to serve as a blank. The prepared 96-well plate was inserted into the instrument and UV absorbance was measured using the following settings.

Instrument settings

	SPECTROstar® Nano	FLUOstar®/ POLARstar® Omega	CLARIOstar®	PHERAstar® *FS*
Detection mode	Absorbance			
Spectro-meter settings	Select the wavelengths: 260 and 280 nm or Measure a spectrum between 220 and 400 nm			
Predefined Protocols available	√	√	√	√

There are two possibilities for the measurement. You can either choose to select up to eight specific wavelengths (in this case wavelengths at 260 nm and at 280 nm) or you can measure a spectrum of the sample. An example for a DNA spectral scan using different concentrations of DNA is given in Figure 1.

Rev. 12/2014
Keywords: **A260, DNA quantification, dsDNA , RNA, ssDNA**

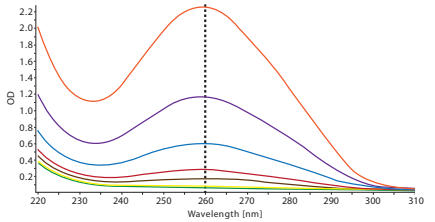


Fig. 1: Absorbance spectrum of different concentrations of calf thymus DNA recorded on the POLARstar Omega. Detection range is between 220 and 310 nm and resolution was set at 1 nm.

Results & Discussion

The data from the measurement was evaluated using the MARS data analysis software from BMG LABTECH. The average value of the blank measurement was subtracted from the measurements made at each concentration and the results plotted. A linear regression fit was performed on the standard values (Figure 2).

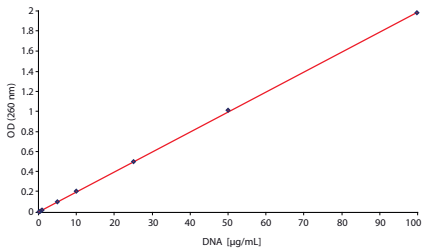


Fig. 2: Linear regression fit performed on the DNA standard curve in the concentration range from 0.1 to 100 µg/mL. An R^2-Value of 0.99988 was obtained indicating a high degree of linearity throughout the concentration range.

The standard curve allows the back calculation of unknown samples. Sensitivity of < 0.3 µg/mL DNA (or about 0.1 µg DNA/well) was observed for measurements with selected 260 nm wavelength and spectrum measurements.

A further option in the new MARS data analysis software is the possibility to determine the DNA concentration of unknown samples without a standard curve. Based on the knowledge that 50 µg of double stranded DNA show an OD value of 1.0, the concentration is automatically calculated without the necessity of pipetting standards into the microplate. It should be taken into account that this method only works well when the path length correction feature is activated.

As double stranded and single stranded DNA or RNA have different extinction coefficients there are different MARS templates available for these different nucleic acids (Table 1).

Table 1: Extinction coefficients of different nucleic acids.

Nucleic acids	Extinction coefficient [cm-1 · M-1]	MARS Data Analysis Software
double stranded DNA	50	dsDNA template
single stranded DNA	33	ssDNA template
RNA	40	RNA template

Conclusion

Because of its spectrometer, BMG LABTECH instruments offer easy handling for DNA absorbance measurements by simply selecting a wavelength of 260 nm or by measuring a spectrum which covers the absorbance maximum. Furthermore, with the help of the data analysis software, MARS, it is possible to determine different nucleic acid concentrations depending on the extinction coefficient.

The ratio of A260/A280 indicates how pure the DNA sample is and it can be measured just as easy and within the same measurement time as A260 alone. A full absorbance spectrum in the range of 220-1000 nm helps to identify impurities and it can be measured within one second per well.

Overview of ELISA assays and NADH/NADPH conversion detection

E.J.Dell and Franka Ganske
BMG LABTECH, Ortenberg, Germany

- **Classic absorbance assays measured using spectrometer-based instruments**
- **Powerful MARS data analysis software utilized to increase assay window**
- **Obtain a full UV/Vis spectrum (220-1000 nm) in less than 1 second per well**

ELISA (405, 420, 450, 492, and 650 nm)

Enzyme Linked Immunosorbent Assay (ELISA) is a commonly used biochemical assay that can detect the presence of an antibody or an antigen in a sample. A sample is incubated with a secondary antibody that recognizes an antigen (or a primary antibody) and that is bioconjugated to an enzyme. This enzyme reacts with a substrate (which can undergo further reactions) producing a solution whose change in absorbance can be measured (Fig. 1)

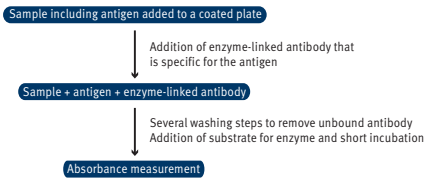


Fig. 1: ELISA Assay Principle.

The two most commonly used bioconjugated enzymes are horse radish peroxidase (HRP) and alkaline phosphatase (AP). AP's substrate, PNPP (p-Nitrophenyl phosphate), absorbs at 405 nm. HRP's substrate is hydrogen peroxide which is coupled with the following chromogens that can be measured with absorption spectroscopy: ABTS (2,2' Azino-di-(3-ethylbenzthiazoline sulfonic acid)) (405-420 nm), TMB (tetramethylbenzidine) (650 nm or 450 nm), and OPD (O-phenylenediamine dihydrochloride) (492 nm).

Instrument Settings

	SPECTROstar® *Nano*	SPECTROstar®/ FLUOstar®/ POLARstar® Omega	CLARIOstar®	PHERAstar® *FS*
Detection mode	Absorbance			
Method	Endpoint			
Optic settings	Select either a full spectrum (220 – 1000 nm) or Select part of a spectrum (350-530 nm) or Select ELISA specific and reference wavelengths, e.g. 405 nm, 450 nm, 492 nm and 650 nm			

The BMG LABTECH´s control software offers pre-defined assay protocols. Just one click is needed to start the measurement.

Mimicking an ELISA assay, yellow dye is used at varying concentrations (Figure 2).

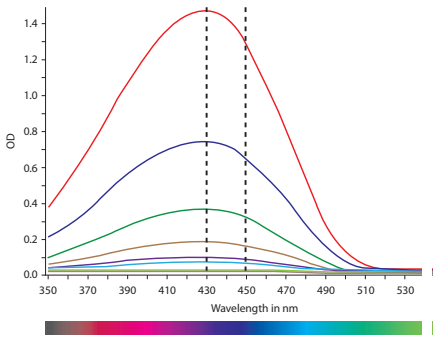


Fig. 2: Spectral absorbance curve of yellow dye which absorbs at similar wavelengths as most ELISA assays (400-500 nm).

Linear regression fit of absorbance measurements at 450 and 430 nm (Figure 3) show that choosing the correct measurement wavelength can increase dynamic range significantly (>13 %).

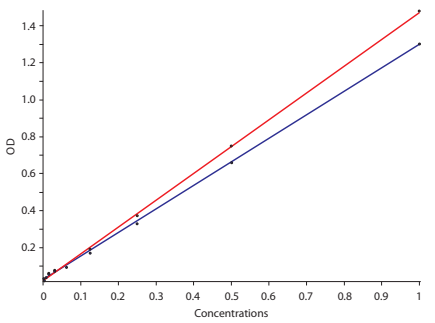


Fig. 3: Linear regression fit of OD measurements at 430 and 450 nm from Fig. 2.

NADH/NAD and NADPH/NADP Conversion (340 nm)

NADH/NAD$^+$ and NADPH/NADP$^+$ are cofactors used by many enzymes in numerous cellular function, including: energy metabolism, mitochondrial functions, calcium homeostasis, oxidative stress, gene expression,

Rev. 12/2014
Keywords: **ELISA, HRP, NADH, NADPH, TMB**

immunological functions, aging and cell death. The reduction of NAD^+ to NADH and $NADP^+$ to NADPH can be monitored at 340 nm because the oxidized forms do not absorb light at this wavelength (Fig. 4)

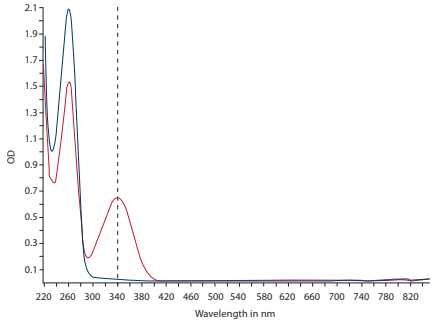


Fig. 4: Absorbance spectra of NAD^+ (blue line) and NADH (red line).

Instrument settings

	SPECTROstar *Nano*	SPECTROstar/ FLUOstar/ POLARstar Omega	CLARIOstar	PHERAstar *FS*
Detection mode	Absorbance			
Method	Endpoint			
Optic settings	Select either a full spectrum (220 – 1000 nm) or Select part of a spectrum (220-400 nm) or Select NADH/NADPH specific wavelength at 340 nm			

Using the MARS Data analysis software, linear regression fits of measurements taken at 260 and 340 nm can be done for $NADP^+$/NADPH or NAD^+/NADH conversion curve (Figure 5). A linear increase in signal at 340 nm is expected (example in Fig. 5 lead to a R^2=0.99). However, unlike the fit of a usual dose response curve, the linear regression fit at 260 nm shows a slight decrease in signal as the NADPH concentration increased (Fig. 5, red line, R^2=0.93). Theoretically, this peak should have no slope since the concentration of the dinucleotide is kept constant. However, there may be a slight decrease because it seems that $NADP^+$ absorbs more light at 260 nm than NADPH. A linear regression fit of the ratioed measurements (340/260 nm) (green line) can be done to correct for this change.

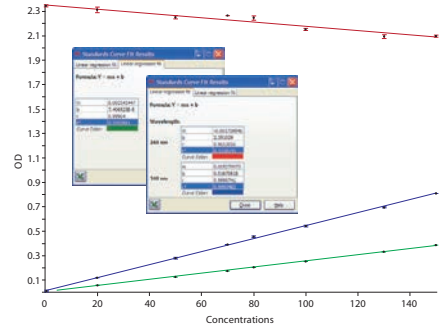


Fig. 5: Linear regression fit of absorbance measurements taken at 260 and 340 nm for a $NADP^+$/NADPH conversion curve. The red line is the fit of measurements taken at 260 nm, the blue line at 340 nm and the green line the ratio of the measurements taken at 340/260 nm.

In Table 1 the sensitivity for NADH and NADPH is shown depending on the number of flashes.

		NADH	NADPH
	Flashes	LOD µM (ng/mL)	LOD µM (ng/ml)
96 well	20	0.748 (497)	1.93 (1610)
	50	0.580 (386)	1.58 (1322)
	100	0.465 (309)	1.11 (931)
384 well	20	3.761 (2501)	1.80 (1497)
	50	3.418 (2773)	1.38 (1151)
	100	3.430 (2281)	1.40 (1169)

Conclusion

The microplate readers from BMG LABTECH all have a UV/Vis spectrometer that can measure any absorbance range from 220-1000 nm at 1, 2, 5 and 10 nm resolution in under 1 second per well. With this flexibility and speed, absorbance assays can be performed easily and fast.

In this application note we have shown the power of the spectrometer in measuring ELISA assays or assays that need the cofactors NAD^+, NADH, $NADP^+$ and NADPH.

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Label-Free SoPRano™ Gold Nano-Rod (GNR) assays on a spectrometer-based microplate reader

E.J. Dell[1], Meike Roskamp[2] and Frederik Van de Velde[2]
[1]BMG LABTECH [2]PharmaDiagnostics

- **The SoPRano™ label-free assay requires full spectral analysis**
- **Easily measure the SoPRano™ label-free LSPR signal with a BMG LABTECH spectrometer-based microplate reader**
- **BMG LABTECH luminescence reader used to monitor dose-dependent responses**

Introduction

Label-Free SoPRano™ Gold Nano-Rod (GNR) kits enable users to design and run label-free, microplate-based homogenous assays for high quality protein-protein interaction analysis based on Localized Surface Plasmon Resonance (LSPR). The interaction of a ligand with surface immobilized protein causes a concentration dependent and highly reproducible redshift of the LSPR peak of the GNR. Using spectrometer based microplate readers from BMG LABTECH, full spectra were captured and the specificity of the signal is demonstrated here. Human Serum Albumin (hSA) and Bovine Serum Albumin (bSA) were separately conjugated to the SoPRano™ GNRs and their respective monoclonal antibodies were bound at various concentrations.

Assay Principle

The SoPRano™ platform enables the monitoring of binding events (for example between two proteins) based on the detection of refractive index changes. This LSPR (localized surface plasmon resonance) based technology is an adaptation of the widely-used SPR technology.

How it works:

- Conjugate your protein to the gold nanorods (GNRs).
- Capture the full absorbance spectrum of the GNR-protein conjugate to determine the λmax, since each GNR-protein conjugate will be different.
- Add the protein ligand to the GNR-protein conjugate and measure a full spectrum. A dose-dependent red shift in λmax will occur upon specific interaction of the protein ligand with the conjugated protein.

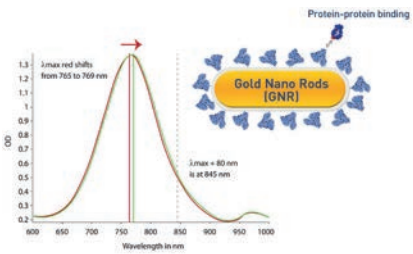

Fig. 1: Gold Nano Rods have an LSPR signal that can be detected with full spectral absorbance. The LSPR peak red-shifts upon ligand binding to the GNR surface, which is instantly captured by the spectrometer.

- This change in the spectrum upon interaction is due to a change in local refractive index at the GNR surface and is very reproducible.
- For kinetic measurements, full spectrum were taken every ten seconds for 3000 seconds. For slow kinetics, measurements may need to be taken for a longer time period; and for fast kinetics, measurements may need to be taken more frequently.

Materials & Methods

- bSA, hSA and their corresponding monoclonal antibodies were purchased from Abcam (United Kingdom) SoPRano™ Kit was from Pharma Diagnostics
- SoPRano™ Kit was from Pharma Diagnostics (Belgium)
- Non-binding, 384-well, black microplates with clear bottoms from Greiner (Germany)

bSA and hSA were separately conjugated to Gold Nano Rods (GNRs) via the proteins' free amine using EDC and sulfo-NHS (see Figure 2).

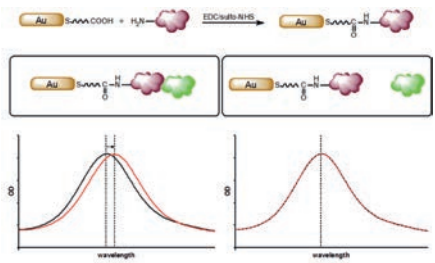

Fig. 2: Proteins are conjugated to Gold Nano Rods via the free amine of the protein using EDC and sulfo-NHS. The graphs show how the λmax red-shifts upon conjugation.

Increasing amounts of the antibodies (5 µL of a 16x concentration) were incubated with the conjugated GNRs (40 µL) for 50 minutes (35 µL of MES buffer was used to bring final volume to 80 µL), followed by full spectrum measurements. Using the MARS data analysis software and calculation templates, ratiometric analysis was easily done (ΔRu = λmax +80 nm/ λmax) and Kds were determined that correspond to other methods of analysis.

Results & Discussion

Using a one-site binding model, dose response curves were produced for hSA-GNRs and its antibody (Figure 3) and for bSAGNRs and its antibody (Figure 4).

Rev. 12/2014
Keywords: **Binding studies, Gold Nano Rods, Protein-protein interaction, Soprano, Spectral analysis**

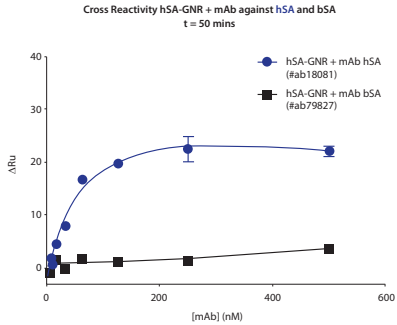

Fig. 3: Dose-response curve shows that hSA's antibody specifically binds to hSA conjugated GNRs, whereas the bSA antibody does not.

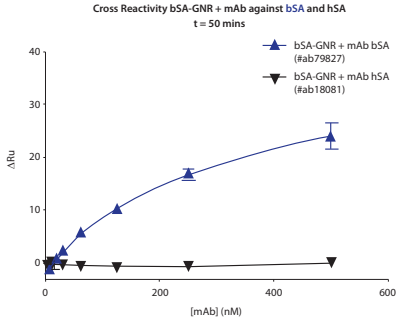

Fig. 4: Dose-response curve shows that bSA's antibody specifically binds to bSA conjugated GNRs, whereas the hSA antibody does not.

Using the opposite antibody for that system, cross-reactivity was shown not to occur. Signal to noise ratios (S:N) of more than 24 and Z'-factors greater than 0.80 prove this to be a robust and reproducible system. Furthermore, calculated Kds correspond with the expected results.

In figure 5, kinetic curves of hSA-GNRs binding to different concentrations of an hSA antibody were created from full spectral measurements. Using a one-site hyperbola binding fit and the last kinetic cycle when equilibrium is reached, a dose response curve can be created which gives the binding constant KD (not shown). The kinetic measurements were used to produce association (ka) and dissociation (kd) rates via a binding kinetic equation in the MARS Data Analysis software.

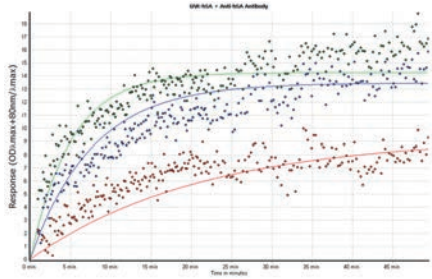

Fig. 5: Human serum albumin was coupled to the SoPRano™ gold nanorods. An antihuman albumin antibody was tested for binding. MARS Data Analysis software was used to produce binding kinetics from the spectral kinetic data.

Conclusion

This label-free SoPRano™ platform from Pharma Diagnostics enable high-throughput, plate-based, homogeneous LSPR assays to be performed for the determination of protein-protein interactions. The spectrometer-based microplate readers from BMG LABTECH capture a full spectra in less than one second per well. Full spectra are needed for the SoPRano™ assay to determine the shift in the λmax upon binding. Subsequently, the MARS data analysis software easily allows for the ratiometric analysis that is needed for this assay.

DNA measurements in low-volume samples, microplates and cuvettes

Mark Gröne, Franka Ganske, Catherine Wark and EJ Dell
BMG LABTECH, USA

- **LVis Plate for sample volumes as small as 2 µl**
- **Sensitivity for cuvette- and microplate-based measurements presented**

Introduction

Absorbance measurements

Definition absorbance

Generally most substances in solution are able to absorb light at a special wavelength or wavelength range. This means that after sending a defined amount of light (I_0) through the solution, a reduced amount of light (I) will be detected afterwards.

$$\text{Absorbance (A)} = \log\left(\frac{I_0}{I}\right)$$

There is a direct linear relation between the absorbance and the concentration of the solute, up to certain limits. This relation is shown in the Beer-Lambert or Beer's law.

$$\text{Beer's law: } A = b \cdot c \cdot \varepsilon$$

b = pathlength [cm]
c = concentration of absorbing substance in solution [mol/l or M]
ε = substance-specific constant [cm-1 M-1] (extinction coefficient)

In this technical application note the direct determination of DNA using absorbance is explained.

DNA

Present in all living organisms, DNA nucleotides consist of a sugar backbone, a base (thymine, guanine, adenine, cytosine) and a phosphate group. The nitrogen rich bases absorb light at 260 nm, this wavelength can be used to determine the DNA concentration. As DNA is so highly studied, the extinction coefficients for dsDNA, ssDNA and for RNA are widely known. The reciprocal value of the coefficient at a 1 cm pathlength can be used as a factor to determine the concentration of nucleic acids.

Table 1: Reciprocal value of extinction coefficients for nucleic acids.

Nucleic acids	1 / Extinction coefficient [µg/mL][1]
double stranded DNA	50
single stranded DNA	33
RNA	40

[1]Using a 1-cm pathlength of light, the extinction coefficient for nucleotides at 260 nm is 20 per cm per M. Based on this the absorbance at 260 nm in a 1-cm quartz cuvette of a 50 µg/mL solution of double stranded DNA, 33 µg/mL solution of single stranded DNA or a 40 µg/mL solution of single stranded RNA are all equal to 1 OD.

The extinction coefficients enable DNA measurements without preparing a standard curve.

Pathlength Correction

With the SPECTROstar® *Nano* it is possible to measure in cuvettes and also in microplates. Cuvette based measurements show the advantage that all absorbance values are automatically normalized to 1 cm. These values can be directly used to calculate the DNA concentration using Beer's law (equation 2) and known extinction coefficients (Table 1).

In microplates the pathlength will vary, depending on the volume of liquid in the well along with the height and dimensions of the well (i.e. 96- versus 384-well plates). To obtain data that can be used in Beer's law it is necessary to normalize the absorbance results to a 1 cm pathlength (b in equation 2). Pathlength correction can be achieved through a number of methods:

- Use a microplate with a defined pathlength (e.g. LVis Plate, pathlength = 0.5 mm). OD measurements are taken and a standard multiplication value is then applied to achieve a 1cm pathlength.
- Use of the pathlength correction feature in the software for standard microplates. The volume and microplate used is specified in the test protocol and an algorithm will then be applied to the data.
- Use a known water peak value correction to normalize the data.

$$\text{Pathlenght of Sample} = \frac{\text{(A977-A900) Sample}}{\text{(A977-A900) 1 cm (Water/Buffer*)}}$$

*= Correction factor

One advantage of using a water peak correction over other methods is that a pathlength can be created for each well allowing different volumes to be dispensed into one microplate if needed, more importantly this method will also correct for pipetting errors that may occur during sample preparation.

Purity Determination

DNA samples can contain impurities that will affect the values at 260 nm. Therefore it is recommended to also measure wavelengths where impurities have an absorbance maximum:

1. Contamination by protein (280 nm)
2. Contamination by phenolate, thiocyanate (230 nm)
3. Scattering of light caused by particulates (340 nm)

A common purity check is to calculate the 260/280 ratio in order to look for protein contaminations. Pure DNA gives a ratio between 1.8 – 2.0, whereas pure RNA shows ~ 2.0. High quality samples should be above 1.7. Another commonly used step is to correct OD values for scattered light by subtracting the OD value at 340 nm from the OD values at 260 or 280 nm, this greatly reduces the standard deviation and increases the sensitivity of measurements.

Rev. 05/2007
Keywords: **A260, Cuvette, DNA quantification, Low-volume**

Materials & Methods

- UV-Cuvettes half semi-micro from Brand
- UV Star 96-well plate from Greiner
- Black 384-well LoBase plate µClear, COC with UV bottom from Greiner
- SPECTROstar *Nano* equipped with LVis Plate

Herring Sperm dsDNA and HPLC grade water was obtained through normal distribution channels.

Results & Discussion

DNA measurements using the LVis Plate

The LVis Plate is a special microplate that contains 16 sites suitable for measuring 2 µL samples. After pipetting onto the microdrop well on the LVis Plate, the lid is closed resulting in a pathlength of 0.5 mm being generated for all 16 sites. A dsDNA standard curve for measurements with the LVis Plate is shown in Fig. 1.

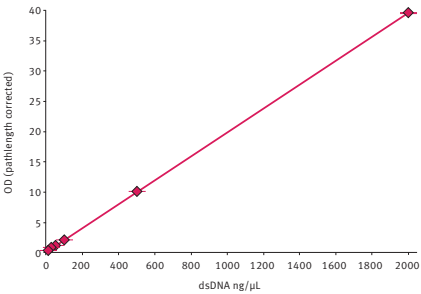

Fig. 1: DNA standard curve obtained using the LVis Plate.

Sensitivity values (LOD) will depend on how reliably the blank can be measured. The DNA sensitivity for the LVis Plate was calculated to be <2 µg/mL.

DNA measurements using a standard microplate

In microplates the best sensitivity is achieved when the highest possible volume is used generating a longer pathlength. This is OK generally but if sample is limited then users will usually want to use as low of a volume as possible. Therefore, it is necessary to find the best compromise between volume and sensitivity. Table 2 shows the limit of detection obtained for different volumes in different microplates. The data presented in Table 2 further indicate that correcting the data at 340 nm generally leads to a higher sensitivity.

Table 2: dsDNA sensitivity in 96-well and 384-well plates.

Plate Format	Volume	LOD in µg/mL based on A_{260}	LOD in µg/mL based on A_{260}-A_{340}
96	350 µL	0.39	0.24
96	300 µL	0.22	0.20
96	200 µL	0.45	0.36
96	100 µL	1.03	0.82
96	50 µL	1.3	1.3
384	20 µL	1.9	0.53
384	5 µL	2.8	2.4
384	3 µL	2.7	2.4

DNA measurements using a cuvette

Measuring the DNA content in cuvettes is still common in labs if only a limited number of samples need to be determined. Cuvette measurements have the advantage that the resulting absorbance values are already normalized to 1 cm. Fig. 2 shows results from dsDNA measurements in cuvettes.

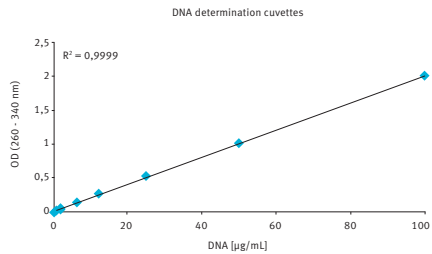

Fig. 2: DNA standard curve obtained in cuvettes.

As shown in Figure 2, a high linearity (R^2 = 0.9999) in the low DNA concentration range is achieved in cuvettes. A blank correction and A_{260} - A_{340} referencing is recommended to obtain the highest sensitivity (< 0.2 µg/ml).

Conclusion

There are different possibilities to measure DNA samples using the SPECTROstar *Nano*. If there is enough DNA material available and only a few samples that should be measured, cuvette measurements can be performed. For higher throughput a microplate should be used. If sample volume is very limited, then the LVis Plate is recommended as it offers a great linear range, a high sensitivity and only 2 µL of sample is needed.

SPECTROstar® *Nano*

Authentication and quality testing of distilled spirits using the SPECTROstar® *Nano*

Gary Spedding[1] and Carl Peters[2]
[1]Brewing and Distilling Analytical Services, LLC, Lexington, KY, USA [2]BMG LABTECH, Cary, NC, USA

- **UV-Vis scanning and 'fingerprint' detection enables quality testing and authentication of distilled spirits**
- **SPECTROstar® *Nano* rapidly and efficiently measures multiple samples of distilled spirits**
- **Allows distillers to detect counterfeits and track adulteration**

Introduction

The distilled beverage industry is creating new products at a rapid pace; at the same time it is under constant attack from counterfeiters and those who would adulterate or dilute distilled spirits and liqueurs. Recently, it has been discovered that UV-Visible spectroscopy provides a rapid and powerful tool to test the quality of distilled spirits and to detect adulterated and counterfeit products[1-5].
Distilled spirits are broadly classified as white and aged spirits. As spirits age in the wood they pick up sugars, tannins, other complex biochemical components and color and undergo considerable chemical reactions. This changes the profile of the spirit in complex and still largely unresolved ways. In addition, for some spirit designations coloring, sugar, citric acid and botanicals may be present. Changes in aging and the addition of components may be followed or detected through uv-visible scanning of samples.
The SPECTROstar *Nano* microplate reader can rapidly and precisely measure the spectra of multiple samples at a time. The integrated spectrometer can take spectra over time and the MARS data evaluation software shows overlay plots of all measurements allowing near-real-time evaluation of distilled spirit quality.

Assay Principle

Distilled spirits should be examined undiluted and are tested using small volumes which allow for very short path-lengths; an important criterion when measuring darker colored or more complex spirits and liqueurs.
For purposes of authentication a data library of specific style and brand "fingerprints" is acquired and stored within the MARS software. It is then a simple matter of calling up the appropriate data set to compare with newly acquired sample data and render "go-no go", recall decisions based upon the results obtained.
Typically spectra are recorded from about 250 to 400 nanometers and a notable feature for many spirits is a broad band centered about 280 nanometers, whose amplitude changes according to the type of spirit and brand. Unique fingerprints are defined both from within the same style, and with brand differences depending upon process, blending, additives and aging. Over time a library of product fingerprints can be generated which can help in maintaining product quality, and detecting adulteration of the product.

Materials & Methods

- UV transparent 96-well plates (Costar)
- SPECTROstar *Nano* (BMG LABTECH)

The indicated spirit samples (Figures 2-4) were pipeted into microplates in replicates taking care not to introduce bubbles. Low, 100 µl, volumes were employed and the plates read on a SPECTROstar *Nano*.

Instrument settings

Measurement mode:	Endpoint
Wavelength Settings:	Spectra
Wavelength Range:	220-1000 nm
Scan Resolution:	2 nm
Path Length Correction:	Off

Results & Discussion

The typical spectral profiles for several types of distilled spirits are presented in Figure 2. The spirits represented a range from white colored silver tequila to aged tequila and through darker colored spirits such as Canadian whiskey, Scotch whisky and three brands of Bourbon whiskey. Thus it is seen that there are broad spirit class distinctions and subtle yet unique distinctions between the same type of spirit.

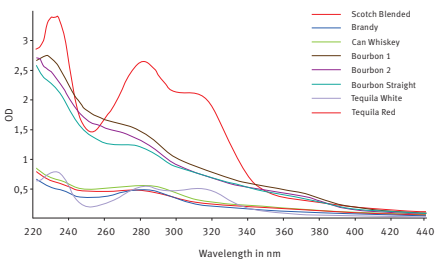

Fig. 2: UV-Visible fingerprint profiles of white and colored classes of distilled spirits.

The distiller is often interested in the desirable aging changes associated with maturation in the wood. While this is a complex topic it is noted again that profile changes can be rapidly monitored by selected timings and spectral analysis (Figure 3). A white whiskey and an un-aged cachaça show a typical low amplitude un-aged profile. The same distillery for the cachaça also produces a classic wood aged product and shows the enhanced amplitude profile typical of such wood aging and is also distinctive for the brand. Furthermore the fingerprint is valid across batches as witnessed by the two aged profiles. The profiles are similar enough to warrant the finding that the uv-visible scanning method is generally valid for detecting and authenticating brands.[3]

Rev. 01/2015
Keywords: **Adulteration, Quality control, Spectral analysis, Spirits, UV absorbance**

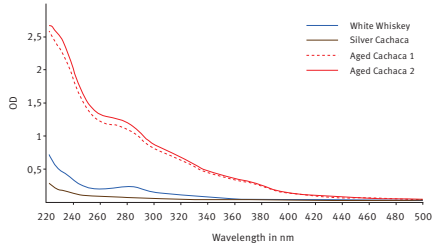

Fig. 3: UV-Visible fingerprint profiles of a white whiskey and un-aged and an aged Cachaça (cane sugar spirit).

Certain spirits are allowed to have a spirit-grade caramel added. The quality of the caramel may be detected in part by spectroscopy (Figure 4). Such information is useful in declared caramel addition or its suspected addition when not expected. Other additives may also be detected in a similar way.

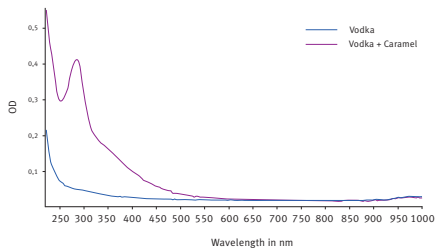

Fig. 4: UV-Visible fingerprint profiles of a white spirit "as is" and after the addition of a spirit grade caramel.

Conclusion

We have shown here the power of UV-visible spectroscopy using the SPECTROstar *Nano* in quality control testing of distilled beverages. It is useful in brand authentication and in counterfeit and adulterant studies. Unlike other instruments on the market the SPECTROstar *Nano* is not dedicated just to this type of work; it can handle multiple samples at a time and can return data on multiple samples in minutes – the same time for one sample by other instruments dedicated to just this application. No sample preparation or dilution is necessary, no blanks are needed and no switching of path-length devices are required making for fast, efficient and precise data collection. Sample size can be as little as 100 microliters and replicate analyses are easy to test in the multi-well, microplate format.

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5. Nascimento, E. C. L., et al. (2011) J. Braz. Chem. Soc. **22**: 1061-1067.

ProteaseTag™ active NE immunoassay: a rapid test to quantify neutrophil elastase levels in patients

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- **Coupled with a specific immunodetection step the ProteaseTag™ activity-dependent immunoassay provides a selective and quantitative measurement of active NE levels in patient sputum samples**
- **CLARIOstar® was used to spectrally analyse the results and automatically return quantified data**

Introduction

Respiratory diseases such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) involve cycles of chronic infection and prolonged inflammation of the airways, leading to poor lung function. Antibiotics and anti-inflammatory treatments have been shown to help patients in these cases.[1] In both CF and COPD, neutrophils drive the inflammatory response,[2,3] and the presence of neutrophil elastase (NE), a protease secreted by neutrophils, has been established as a key biomarker of infection and inflammation.

The detection of NE can therefore be used to monitor the disease status of the patient as it correlates with the occurrence of acute episodes of disease activity.

Established methods for the quantitation of active proteases are based on chromogenic or fluorogenic peptides that, in complex biological samples (such as sputum), can be broken down by other activities (either host or bacterial in origin) and therefore lack the necessary specificity for accurate quantitation. Traditional immunoassays do not distinguish between the active and inactive forms of the enzyme thus do not accurately map the severity of disease.

In this application note, the Active NE ProteaseTag™ kit is shown to detect and quantify levels of NE in patient samples. ProAxsis' ProteaseTags™ are engineered to inhibit and bind to specific proteases. Once bound, they form a bridge to a solid support allowing their incorporation into an immunoassay format for specific quantification.

Assay Principle

The ProteaseTag™ Active NE Immunoassay captures active NE using NE-Tag. (Fig.1) A subsequent antibody step provides additional signal amplification with increased sensitivity. Detection is achieved within an ELISA-type format and provides results in less than 3 hours. Detection with NE-Tag is quantitative and discriminatory. The assay kit is suitable for the measurement of the following:

- sputum sol
- bronchoalveolar lavage
- wound exudate
- serum free cell culture

The detection range of the assay is 1000-15.6 ng/mL and a sensitivity of 3.3 ng/mL.

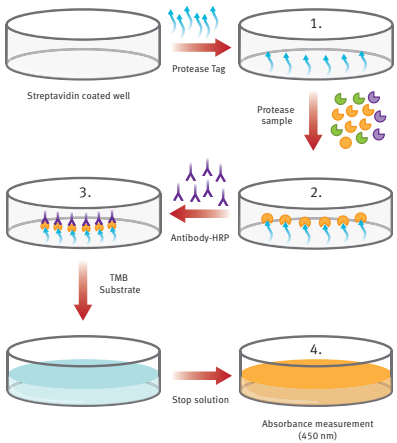

Fig. 1: Procedure overview of ProteaseTag™ Active Neutrophil Elastase Immunoassay.

Summary of the procedure

1. Coat plate with NE-Tag. Wash.
2. Add standards and samples. NE-Tag captures active NE from the sample, inactivating and stabilizing the enzyme. Wash.
3. An antibody-conjugate step enables detection of the complex by the subsequent addition of colorimetric substrate/stop solution.
4. Measure the plate in absorbance mode at 450 nm.

Materials & Methods

- ProteaseTag™ Active Neutrophil Elastase Immunoassay (PA001) from ProAxsis
- CLARIOstar microplate reader from BMG LABTECH

Prior to the assay, sputum samples were collected from individuals with CF and briefly processed. All standards and samples were kept on ice before addition to the microplate well. All other reagents were brought to room temperature before addition.

Standards were diluted and the plate prepared according to the manufacturer´s instructions and samples, standards and blanks added to the plate in duplicate.

Instrument settings

Measurement Type:	Absorbance
Reading Mode:	Endpoint
Wavelength:	Spectra or Discrete wavelength at 450 nm

Rev. 02/2016
Keywords: **Biomarker, ELISA, Immunoassay, Inflammation, ProAxsis**

Settling time:	0.5 sec
Shaking settings	
Shaking frequency [rpm]:	300
Shaking mode:	double orbital
Shaking timing:	3 sec before plate reading
Temperature:	37°C

Results & Discussion

First, the spectral profile of the standards was analysed (Fig. 2). This confirmed that 450 nm was the optimum wavelength at which to read the absorbance.

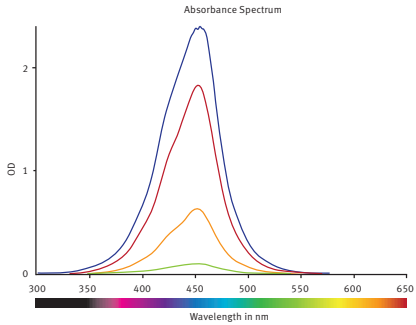

Fig. 2: Absorbance spectra of samples containing hNE at different concentrations. The values are corrected for the blank.

Initial raw data was blank corrected using the MARS Data Analysis software. A standard curve was generated with the help of the standard curve wizard. The hNE concentration from each sample was then automatically calculated from the regression curve (Fig. 3).

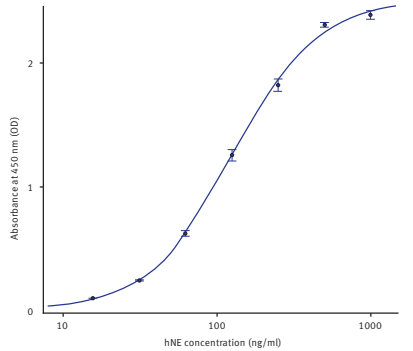

Fig. 3: hNE standard curve using hNE standards provided with the kit.

Conclusion

The ProteaseTag™ Active NE Immunoassay provides a simple and quick method of assessing active neutrophil elastase levels in a given sample. Because the assay accurately quantifies active protease levels, the kit constitutes a reliable method for the detection of infection and inflammation in clinical samples. In addition to airways samples, active NE has been quantified in a number of other biological samples such as wound fluid. The kit significantly correlates with other activity-based methods for the detection of active NE but has greater selectivity and specificity than peptide-based substrates when used with complex biological samples. This can be of significant impact when determining relationships or associations between a protease biomarker and other markers of disease.

The CLARIOstar provides a platform on which to read and analyse data to completion, resulting in a fast and easily reproducible method for running the assay.

References

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Fluorescence analysis of reactive oxygen species (ROS) generated by six isolates of *Aspergillus fumigatus*

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- **Detection of intracellular H_2O_2**
- **LOD of 5 nM H_2O_2 in 200 µL**
- **LOQ of > 10^7 cells per mL**

Introduction

Reactive oxygen species (ROS) are essential intermediates in oxidative metabolism. Nonetheless, when generated in excess, ROS can damage cells by peroxidizing lipids and disrupting structural proteins, enzymes and nucleic acids. Excess ROS are generated during a variety of cell stresses, including ischemia/reperfusion, exposure to ionizing and ultraviolet radiation and/or inflammation. ROS may contribute to inflammation and tissue damage.

The processes leading to ROS generation can be monitored using luminescence analysis or fluorescence methods. Intracellular ROS generation in cells can be investigated using 2',7'-dichlorfluoresceindiacetate (DCFH-DA), which is an established compound to detect and quantify intracellularly produced H_2O_2.
The conversion of non-fluorescent DCFH-DA to the highly fluoresecent compound, 2',7'-dichlorfluorescein (DCF), happens in several steps. First, DCFH-DA is transported across the cell membrane and deacetylated by esterases to form the non-fluorescent 2',7'-dichlorfluorescein (DCFH). This compound is trapped inside of the cells. Next, DCFH is converted to DCF through the action of peroxide, which is generated by the presence of peroxidase.

Aspergillus species are of interest in the pathogenesis of several dermatological diseases. It is uncertain whether *Aspergillus* itself may generate ROS and therefore actively induce tissue damage. The present study investigates whether *Aspergillus* species are capable of producing ROS by themselves and if there are differences between the several strains.

Materials & Methods

Six isolates of *Aspergillus fumigatus* (AF 65, AF 71, AF 72, AF 91, AF 210, AF 294) cultured 5 weeks on Sabouraud-Glucose-Agar (BAG) were investigated.
After addition of isotonic NaCl solution and centrifugation with 100 rpm for 10 min, the blastospore concentration could be estimated by counting in CASY 1 (Schärfesystem).
These cell suspensions with concentrations of 10^5 to 10^7 cells/mL were measured on a filter-based BMG LABTECH microplate reader using 100 µL of fungal cell suspension after incubation with 100 µL DCFHDA (0.4 nM) for each single test. To eliminate LBS induced effects, polymyxin B (3 mg/mL) was added to all experimental suspensions.

Each measurement was done at least sixteen times in duplicate for calculation of the mean and the standard error of the mean.

Results & Discussion

The ability of various *Aspergillus* species to generate ROS was investigated.

For all fungal cells, a linear increasing fluorescence activity could be observed depending on the incubation time with DCFA-DA. By using a calibration curve, the measured fluorescence signals were converted to H_2O_2 concentrations and one example is given for AF 71 with different incubation periods (Fig. 1). Because of the small sized *Aspergillus fumigatus* (2-3 mm), detectable fluorescence was observed only at concentrations >10^5 cells /mL.

The ROS generation showed a linear and direct proportional dependence on cell numbers and the results were reproducible on 3 different days with a fixed incubation period of 2.5 hours at 37°C (Fig. 2). The highest value could be found at concentrations of 10^7 cells /mL.

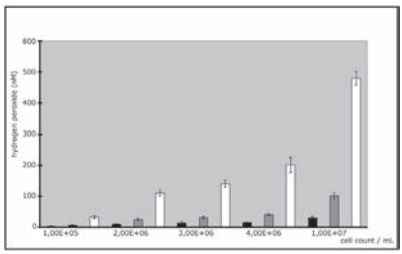

Fig. 1: H_2O_2 production of AF 71 depends on incubation period and cell number.

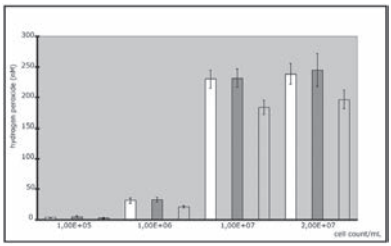

Fig. 2: H_2O_2 production of AF 71 depends on cell number with 4 hr incubation time at 37°C on three separate days.

Rev. 06/2004
Keywords: **2',7'-Dichlorfluorescein-diacetate, *Aspergillus*, DCFH-DA, H_2O_2, ROS**

The isolates of *A. fumigatus* AF 91 and AF 72 are resistent against itraconazole (antifungal agent) and AF 65 is resistant to amphotericin B (antifungal agent). We investigated whether or not there are connections between the resistance and the ROS generation. Interisolate differences could be found (Fig. 3).

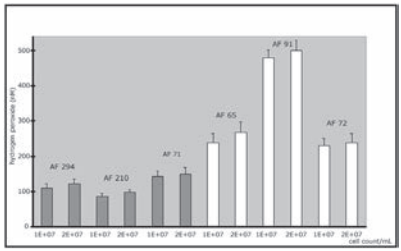

Fig. 3: H_2O_2 production of several strains of *Aspergillus fumigatus* with 3 hr incubation time at 37°C. The last three strains are resistant against antifungal agents.

Conclusion

The morphological event of fungi, usually acknowledged as a major factor of virulence, is associated with increased intracellular ROS formation, which are most likely secreted. Together with phospholipases, ROS are capable of destroying the host cell membranes. This process may contribute to the invasiveness of *A. fumigatus* and to the inflammatory response of the host.

Clearly, the pathogenicity of *Aspergillus* species is a function of a multitude of parameters working together in a sequential and cooperative manner to establish infection.

It has been shown by several authors, that water as well as superoxide, hydrogen peroxide and OH-radicals can be generated in the course of the mitochondrial electron transport process. Fungi also possess the normal and the alternative pathway of electron transport. The interesting connection between the monovalent oxygen reduction and the energy conservation in isolated chloroplastes was described. The hydrogen peroxide formation at the phosphorylation points I and II is disposed by a cytochrome c oxidation process.

In this study, the method of ROS fluorescence measurement was utilized on different unstimulated *Aspergillus* species for the first time. There were linear correlations found between ROS levels and blastospore concentrations. A pathophysiological meaning of the released oxygen metabolites as an additional factor of virulence in the complicated system of inflammatory reactions, which was also estimated in Saccharomyces cerevisiae, is hence not to be excluded.

Use of CyDye fluors for improved FRET protease assays on a BMG LABTECH fluorescence microplate reader

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- **Improved assay platform with BMG LABTECH readers and CyDye™ labelled peptides**
- **Enhanced and significant fluorescence increase compared to traditional fluors**
- **Development of CyDye™ labelled substrates to control MMP activity**

Introduction

Members of the matrix metalloproteinase (MMP) family play an important role in tissue remodeling and repair. However, inappropriate MMP activity has been implicated in a number of disease states including arthritis, tumour invasion and metastasis, and cardio-vascular disease. The development of agents to control MMP activity continues to be a major focus for the pharmaceutical industry.

Many synthetic MMP peptide substrates have been described which incorporate a fluorophore and a quencher moiety positioned on either side of the enzyme cleavage site. We have prepared a number of CyDye™ labelled MMP substrates. Cleavage of these substrates with specific MMP enzymes produces improved and significant fluorescence increase at donor fluorophore wavelengths (Figure 1), when compared to traditionally used fluors.

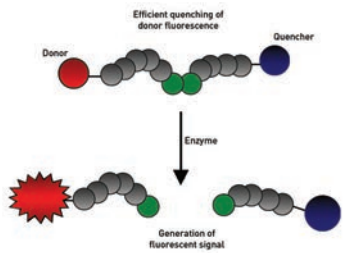

Fig. 1: FRET protease assay (schematic).

The POLARstar® Omega and PHERAstar® *FS* have 2 Photo Multiplier Tubes (PMT) and hence do not have to switch filters or polarizers to read the second channel, Simultaneous Dual Emission (SDE) makes assays like FRET faster and more precise.

Material & Experimental

Peptide Substrates:

Peptides were synthesized and labelled with active CyDye esters (Amersham Biosciences) using standard synthesis and labelling procedures. Following purification by RP-HPLC and confirmation of purity by mass spectrophotometry, dual labelled peptide was freeze-dried. Prior to use, labelled peptide substrates (Table 1) were dissolved in DMSO and stocks were stored at -20°C. Peptide Mca-PLGL-Dpa-AR-NH2, supplied as a freeze-dried powder (CN Biosciences), was dissolved in methanol as per manufacturer's instructions.

Table 1: Peptide sequences for cleavage assay

Peptide I	Cy3B-PLG↑ LAARK-Cy5Q
Peptide II	Cy3B-PLG↑ LFARK-Cy5Q
Peptide III	Mca-PLG↑ L-Dpa-AR

Mca, (7-Methoxycoumarin-4-yl)acetyl
Dpa, 3-(2,4-Dinitrophenyl)-L-2,3-diaminopropionic acid

Activation of MMP-2:

Human recombinant MMP-2 pro-enzyme (CN Biosciences) was activated by incubation in assay buffer containing 4-aminophenyl mercuric acetate for 2 hours at 37°C.

Standard Assay:

Labelled peptides (400 nM) were incubated at 37°C with or without activated MMP-2 enzyme in assay buffer (50 mM Tris pH 7.5, containing 150 mM NaCl, 10 mM $CaCl_2$, 10 µM $ZnCl_2$, 0.05% (w/v) Brij™-35 and 0.05% NaN_3). Assays were configured in black, opaque 384-well plates (Greiner) in final reaction volumes of 60 µL. Plates were read on BMG LABTECH fluorescence plate reader using 320/390 nm and 530/570 nm excitation and emission wavelengths for Mca and Cy™3B respectively.

Results & Discussion

We have prepared two MMP substrates that contain Cy3B and Cy5Q as a fluorophore/quencher pair. In a series of experiments, these were compared with each other and with the well characterized peptide Mca-PLGL-Dpa-AR. A time course for hydrolysis of the substrates by MMP-2 was established (Figure 2) and all three peptides were found to be effectively cleaved.

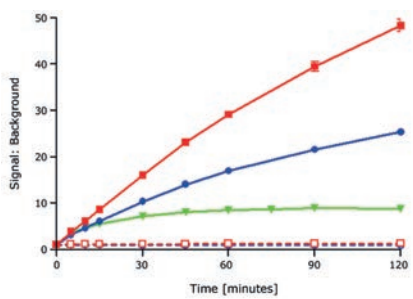

Fig. 2: Time course analysis of MMP-2 cleavage. Peptides I (●), II (■) and III (▼) were incubated at 37°C with (solid symbols/lines) or without (open symbols/dashed lines) 4ng/well MMP-2 as described and fluorescence signals were measured over time using the BMG LABTECH reader. Values are plotted as means ± SD (n=3).

Rev. 05/2007
Keywords: **Cy3, Cy5, CyDye, Matrix metalloproteinase, Protease, Quencher**

At the assay endpoint we calculated signal/background (S/B) values for the three peptides and the recognized Z' statistical factor for peptides I and II (Table 2). Overall, results showed that peptide II was the more favourable substrate for MMP-2.

Table 2: Summary statistics for MMP-2 assay.

	BMG POLARstar	
	S/B	Z'
Peptide I	25:1	0.95
Peptide II	49:1	0.93
Peptide III	9:1	nd

Conclusion

We have employed CyDye fluors in a FRET protease cleavage assay for the enzyme MMP-2. Substrates combining the fluorescent donor (Cy3B) with a Cy5Q quencher in a de-quench assay format were compared with an equivalent substrate incorporating a methyl-coumarin fluor and dinitrophenyl based quencher.

All of the peptide substrates (Figure 2) were efficiently hydrolyzed by the MMP-2 enzyme. Signal increases, measured on the BMG LABTECH fluorescence microplate reader, were >25-fold following hydrolysis of the CyDye labelled substrates, compared with only a 9-fold signal increase following hydrolysis of the Mca/Dpa labelled substrate (when evaluated by time course analysis). For both of the CyDye labelled peptides, no significant signal increases were observed in control (no enzyme containing) wells. This combination of CyDye labelled peptides and detection on the BMG LABTECH reader provides an improved assay platform when compared with more traditionally used fluors (such as Mca).

The two CyDye peptides differ at the P2' position (Table 1). Other well characterized substrates contain either tryptophan or Dpa at this site. The data presented here suggest that phenylalanine is also a favourable residue in subsite P2'. This is in line with the observation that MMP enzymes favour aromatic side chains at P2'.

PHERAstar® *FSX*
PHERAstar® *FS*

Omega Series

Ion channel assay development using Invitrogen's FRET-based voltage sensor probes

Randall L. Hoffman, Steve Duff and Naveeda Qadir
Invitrogen Corporation, Madison, WI 53719, USA

- **Used for ion channel assay development**
- **Simultaneous Dual Emission detection of FRET**
- **Injection and FRET measurement at the same time**

Introduction

Ion channels are important drug targets because of their critical role in nerve, cardiac, endocrine and skeletal muscle tissues. The lack of sufficiently sensitive screening systems has hampered research in this area. This application note focuses on Voltage Sensor Probes (VSP), a Fluorescence Resonance Energy Transfer (FRET)-based voltage-sensing assay technology from Invitrogen for measuring changes in cellular membrane electrical potential.

This technology enables detection and measurement of rapid changes in membrane voltage and quickly reports them as fluorescence signals from living cells. The ratiometric method used to detect and quantify changes in cellular membrane potential significantly reduces errors arising from well-to-well variations in cell number, dye loading and signal intensities, plate inconsistencies and temperature fluctuations. These combined features make VSP technology highly amenable for high-throughput screening (HTS) applications.
This application note demonstrates the use of BMG LABTECH microplate readers for development of VSP ion channel assays.

Assay Principle

VSP is a Fluorescence Resonance Energy Transfer (FRET)-based assay technology used for ion channel drug discovery. The FRET donor is a membrane-bound, coumarin-phospholipid (CC2-DMPE), which binds only to the exterior of the cell membrane. The FRET acceptor is a mobile, negatively charged, hydrophobic oxonol [either $DiSBAC_2(3)$ or $DiSBAC_4(3)$], which will bind to either side of the plasma membrane in response to changes in membrane potential. Resting cells have a relatively negative potential, so the two probes associate with the exterior of the cell membrane, resulting in efficient FRET (Figure 1). Exciting the CC2-DMPE donor probe (filter: 400-10 nm) generates a strong red fluorescence signal (filter: 570-20 nm) from the oxonol acceptor probe.
When the membrane potential becomes more positive, the oxonol probe rapidly translocates (on a subsecond time scale) to the other face of the membrane. Thus, each oxonol probe "senses" and responds to voltage changes in the cell.
This translocation separates the FRET pair, so exciting the CC2-DMPE donor probe and generates a strong blue fluorescence signal (filter: 460-10 nm) from the CC2-DMPE probe.

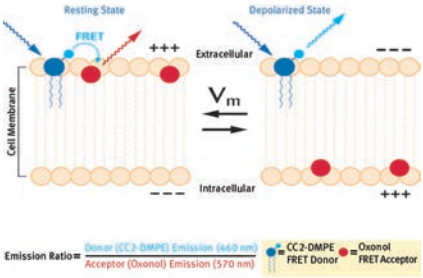

Fig. 1: Schematic illustration of the mechanism of voltage-dependent, FRET-based VSPs.

The ion channel model we have used is endogenously expressed inward rectifying potassium (Kir) channels present in rat basophilic leukemia cells. These channels are opened when high potassium is added to the cell's medium. This causes a change in the cell's membrane potential which can be modulated by barium chloride. In the assay, the cells are manually loaded with the dyes as described below. Barium chloride is then immediately added at various concentrations. After incubation, the plated cells are placed in the reader. The reader begins to take baseline readings at the two wavelengths, injects the high potassium buffer (VSP-2 buffer, below), and continues to take readings for a specified time.

Materials & Methods

Cell Culture

RBL-2H3 (Rat Basophilic Leukemia, ATCC) cells were plated at 50,000 cells/well in Corning® 96-well plates 18-24 hours prior to experimental procedure.

Preparation of VSP Loading Buffers

A. 5 µM CC2-DMPE Loading Buffer: Premix 10 µL of 5 mM CC2-DMPE (Invitrogen) and 10 µL of 100 mg/mL Pluronic® F-127 (Sigma). Add 10 mL of VSP Solution 1 (160 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) and vortex vigorously to mix. Protect from light prior to use.
B. 10 µM $DiSBAC_2(3)$ Loading Buffer: Premix 8.3 µL of 12 mM $DiSBAC_2(3)$ (Invitrogen) and 12.5 µL of 200 mM VABSC-1 (Invitrogen). Add 10 mL of VSP Solution 1 and vigorously vortex to mix. Protect from light prior to use.

Rev. 10/2005
Keywords: **CC2-DMPE, Coumarin, Ion channel, Oxonol, Voltage sensor probes**

Loading Cells

Media were replaced with 100 µL VSP Solution 1 (VSP-1). The VSP-1 was immediately replaced with 100 µL CC2-DMPE Loading Buffer and incubated at room temperature for 30 minutes, covered and protected from light. The CC2-DMPE Loading Buffer was removed after 30 minutes and the plates washed once with 100 µL VSP-1. The VSP-1 was immediately replaced with 100 µL $DiSBAC_2$ Loading Buffer and incubated at room temperature for 30 minutes, covered and protected from light.

$BaCl_2$ (Sigma), when appropriate, was added either immediately following addition of $DiSBAC_2$ Loading Buffer or immediately prior to VSP-2 high K^+ buffer (164.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4).

Results & Discussion

Immediately following the $DiSBAC_2$ Loading Buffer 30 minute incubation, the plates where analyzed the BMG LABTECH microplate reader. Data are analyzed by comparing the baseline subtracted ratio of donor (at 460 nm) to acceptor (at 570 nm) before (Ro) and after (Rf) addition of VSP-2: the final normalized assay ratio = Rf/Ro. A plate readout view is shown in Figure 2A for a "positive control" assay with a 96-well plate with 50,000 RBL cells/well. Column 1 is the no cells control.

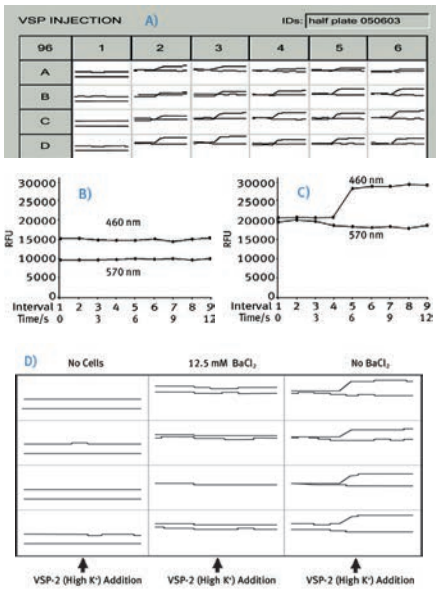

Fig. 2: VSP data on the BMG LABTECH microplate reader.

In Figure 2B a current state window view of a no cell control shows no change in 460 nm upon VSP-2 addition, whereas in Figure 2C an increase in the 460 nm signal upon injection of VSP-2 is detected. Figure 2D shows enlarged views of current state windows from 12 wells of a $BaCl_2$ dose response. Note the lack of 460 nm emission increase in both the cell control and 12.5 mM $BaCl_2$ wells compared to untreated wells.

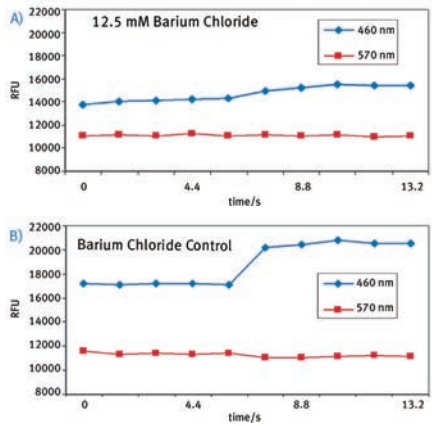

Fig. 3: VSP data on the BMG LABTECH microplate reader.

The raw fluorescent readout was evaluated in Excel and the addition of 12.5 mM $BaCl_2$ avoids a significant change in 460 nm emission (Figure 3A), whereas an untreated control well shows as expected an increase in 460 nm fluorescence signal upon VSP-2 addition (Figure 3B).

Conclusion

This application note demonstrates the compatibility of Voltage Sensor Probe technology with plate readers from BMG LABTECH. The key to the technology is having instrumentation that can measure two wavelengths at the same time. This takes advantage of the ratiometric FRET readout, which greatly reduces variation common to many cell based assays. The ability of the BMG LABTECH readers to inject channel stimulant, switch filters at approximately 1 Hz, and continue to take readings during the kinetic event make these suitable instruments for ion channel assays.

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High-throughput measurement of protein stability using a BMG LABTECH microplate reader

Jean P. Aucamp, Ana M. Cosme, Gary J. Lye and Paul A. Dalby
Dept. of Biochemical Engineering, University College London, London, UK

- **Affordable high-throughput screening method for protein stability**
- **Unfolding transitions observed for cytochrom c and BSA by measuring tryptophan fluorescence**
- **Potential application for the fields of directed evolution, proteomics and therapeutic protein formulation**

Introduction

The measurement of protein stability is essential for elucidating protein function *in vivo*. For example, therapeutic proteins require optimal formulation to improve shelf life and a high-throughput stability measurement would enable many combinations of excipients to be rapidly tested for their effect on protein stability.

Measurements of protein stability are often obtained indirectly by monitoring protein aggregation or residual activity after incubation at elevated temperatures. Both screens rely on irreversible inactivation of the protein upon unfolding. Although these indirect screens have been applied successfully, they may not easily distinguish differences in stability for proteins that spontaneously refold when returned to the activity-assay conditions.

The unfolding transition of proteins can be observed by measuring their tryptophan fluorescence upon perturbation with a chemical denaturant or a temperature shift. Here we describe an unfolding procedure using BMG LABTECH's microplate reader with titrating syringe pump. Protein unfolding transitions were monitored by tryptophan fluorescence at 340 nm, and assessed using bovine and equine cytochrome c (cyt c), as well as bovine serum albumin (BSA) stabilised with various amounts of palmitic acid. Unfolding curves generated by the serial addition of denaturant into single wells, allowed high-throughput stability screens capable of identifying protein variants with unfolding midpoint differences of 0.15 M denaturant concentration or larger. Such a method would be suitable for screening large numbers of proteins or formulation conditions to rank the order of protein stability.

Materials & Methods

Stability measurement

All protein unfolding transitions were measured in F-type, polystyrene, 96-well microplates (Greiner Bio-One) using a fluorescence BMG LABTECH microplate reader with one injector. Protein unfolding was monitored by intrinsic tryptophan fluorescence using a 340 - 10 nm emission filter with excitation at 280 nm.

Protein-unfolding curves were obtained by the serial addition of buffered denaturant stock to 50 µL protein solution per well, containing 20 µg cyt c or 40 µg BSA. Each addition was followed by 30 seconds of mixing by orbital shaking at 350 rpm, and 15 minutes of equilibration prior to taking measurements.

Results & Discussion

The incubation times for each addition of denaturant were optimised to ensure that cyt c and BSA unfolding both reached equilibrium. It was determined empirically that 15 minutes between injections were sufficient for both cyt c and BSA to fully equilibrate, resulting in serial addition experiments that took up to 10 hours for 25 denaturant concentrations. Conformational transition curves for cyt c from bovine and equine heart, are plotted as nine replicates performed in parallel (Fig. 1).

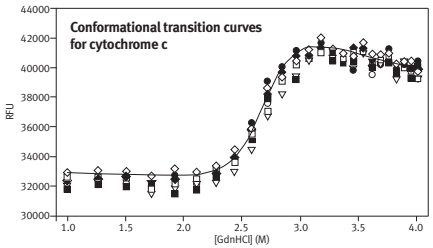

Fig. 1: Conformational transition curves for oxidized cytochrome c (cyt c) with GdnHCl. Serial addition method. Each curve represents data obtained from a single well in which 20 µg cyt c was loaded. Wells were filled in small increments with the appropriate volume of 100 mM Tris, pH 7.0 containing 6.5 M GdnHCl to give the correct final denaturant concentration.

Transition mid-points, C1/2, and mG-values (estimated with non-linear fitting) are summarised in Table 1. The reported stability values are averages of estimates for each of the nine parallel stability analyses.

The inter-run variability was calculated from three separate experiments conducted on separate days, using different stock and protein solutions (Table 1). Mid-points of unfolding for proteins can be estimated to an accuracy of ±0.15 M, but values of mG are significantly overestimated.

Table. 1: Summary of stability data for oxidized cytochrome c (cyt c) from bovine and equine heart.

	$C_{1/2}$ (M)	m_G (kcal/mM)	ΔG (kcal/mol)	%CV		
				$C_{1/2}$	m_G	ΔG
Bovine	2.50 (0.08)[a]	6.19 (1.83)[a]	14.26 (4.60)[a]	2.25[b]	22.66[b]	49.09[b]
Equine	2.67 (0.14)[a]	4.72 (1.19)[a]	11.83 (1.35)[a]	4.73[b]	4.01[b]	6.56[b]

[a] Standard deviations of the population calculated from nine datasets.
[b] Percentage coefficient of variation calculated from twenty-seven datasets.

Rev. 06/2005
Keywords: **BSA, Cytochrome c, Protein stability, Protein folding, Tryptophan**

The ability to rank protein stabilities in terms of $C_{1/2}$ is sufficient for directed evolution screens, where the key requirement is to find enzymes that resist unfolding under the conditions required in a bioreactor. It is also useful for therapeutic formulation in which a combination of excipients is desired that improve the resistance of the protein to unfolding. The large error on m_G-value calculation (6.2 ±1.8 kcal·mol^{-1}·M^{-1} for bovine cyt c) is a result of the inaccurate determination of the pre- and post-unfolding baselines. Unfolding curves of various BSA:palmitate preparations using GdnHCl as denaturant are shown in Figure 2.

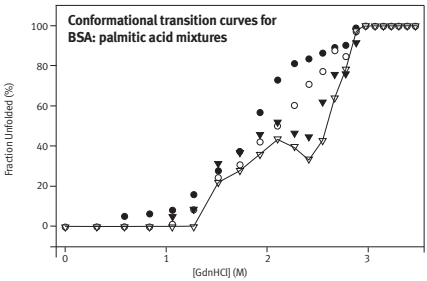

Fig. 2: Conformational transition curves for BSA. 50 µL of a 0.8 mg/mL BSA solution stabilised with palmitic acid at molar ratios of (•) 1:0, (o) 1:3, (▾) 1:5, (∇) 1:6 and buffered with 60 mM sodium phosphate, pH 7.0, were loaded per well. Unfolding was induced by titrating small volumes of a 5.5 M GdnHCl stock solution buffered with 60 mM sodium phosphate, pH 7.0 into each well. Each plot represents data for an unfolding curve obtained from a single well. Data points are interpolated for the highest BSA:palmitate molar ratio (∇), to demonstrate the deviation from a two-state transition.

BSA unfolding curves at high-palmitate concentrations display at least one intermediate in the unfolding pathway, as seen from the deviation from a simple two-state unfolding curve. This intermediate is attributed to the early unfolding of the less stable C-terminal part of the protein. Fatty acid molecules partially stabilise the intermediate, increasing the mid-point of urea induced unfolding.

Three simple numerical methods were used to quantify the stability of the different BSA preparations: i) the unfolding datasets were fitted to a two-state transition model, calculating an approximate estimate for a global $C_{1/2}$-value; ii) the areas below the unfolding curves were calculated using the trapezoidal rule; iii) the denaturant concentration at 60% or 70% unfolding was calculated using a linear equation defined by the two data points either side of the arbitrary set value. Figure 3 depicts the stability estimates using these techniques.

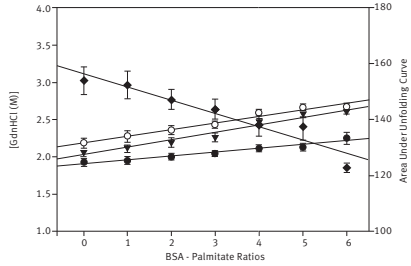

Fig. 3: Stability indexing for palmitate stabilised BSA using different numerical methods. Each dataset in Figure 2 was fit to two-state unfolding. Linear increases in stability are represented by values calculated from: (•) denaturant concentration at apparent midpoint of unfolding; (▾) denaturant concentration at 60 % unfolding; (O) denaturant concentration at 70 % unfolding; (◆) total area under unfolding curve.

All three numerical methods can confidently determine the order of BSA stabilities present at four palmitate concentrations. Interestingly, the fluorescence unfolding curves for BSA in GdnHCl in microplates (Figure 2) can also detect the presence of intermediate states that occur as three palmitate molecules bind to each BSA molecule, i.e. at high palmitate concentrations.

Conclusion

We have developed and characterised an affordable high-throughput screening method for the direct measurement of the stability of proteins in a commercially available microplate reader. The method generates unfolding curves in microwells by autotitration of denaturant and measuring the resulting changes in tryptophan fluorescence. The method is useful when screening for changes in $C_{1/2}$ values of greater than 0.15 M. Combined with a suitable numerical data analysis technique it can be a powerful tool for screening mutant biocatalyst libraries for improved stability.

Protein aggregation monitoring on a BMG LABTECH microplate reader

Stephen Poon and Robert C. Rivers
Department of Chemistry, University of Cambridge, UK

- **Assessment of protein aggregation by absorbance and fluorescence**
- **Assessment of the dose-dependent inhibition of temperature-induced protein aggregation by molecular chaperones**
- **BMG LABTECH microplate readers are well suited for high-throughput protein aggregation screening**

Introduction

Protein aggregation is the underlying cause of many debilitating and often incurable human diseases. Moreover, aggregation during drug formulation invariably leads to reduced efficacy, low yield, poor storage capacity, and increased production costs.

The ability to understand why and how proteins aggregate will undoubtedly help improve upon current strategies aimed at eliminating the causes and effects of this phenomenon. This may sound easy but the reality is that, the conditions required to induce protein aggregation are often highly protein-specific and are as varied as the proteins themselves.

The task of assessing what effect temperature, pH, molecular crowding, sequence mutations, etc. have on the aggregation of a given protein would be almost impossible if not for the availability of firmly established screening techniques.

In this report, we explore some of the features of the BMG LABTECH microplate readers by highlighting examples of their potential uses in the study of protein aggregation.

The results presented here were obtained from recent aggregation studies of a protein which, due to its inherent propensity to self-aggregate to form fibrils, has severely diminished therapeutic potentials. Due to the sensitive nature of this study, it shall be referred in this report simply as "Protein X".

Materials & Methods

1. Assessment of protein aggregation by absorbance and fluorescence

Since the wild-type form of "Protein X" was inherently susceptible to aggregation, we designed a number of variants containing minimal residue substitution. Using the BMG LABTECH microplate reader, we proceeded to test their ability to aggregate in a hope of finding a suitable substitute for the therapeutically valued WT sequence. The time-dependent aggregation by "Protein X" and variants were followed by measuring the turbidity associated with the formation of precipitated protein at A340. The end-point formation of fibrous aggregates was assessed by measuring the fluorescence associated with the specific binding of the dye, Thioflavin T (Th-T), to the ß-sheet-rich fibrils.

Procedure:

i) Prepare "Protein X" at a protein concentration of 1mg/mL

ii) Add 100 mL aliquots to separate wells of a 96- or 384-well microplate, depending on how many tests are required.

iii) Insert the plate into the BMG LABTECH microplate reader and adjust the instrument settings as shown:

For absorbance

- Wavelength: 340 nm
- Meas. start time: 0 s
- Cycle time: 7200 s
- Number of cycles: 30
- Shaking time* : 300 s
- Shaking: orbital
- Temperature: 37°C

For fluorescence

- Ex. filter wavelength: 440 nm
- Em. filter wavelength: 480 nm
- Temperature: 25°C

iv) Start program

* Shaking the samples will help promote protein aggregation and lessen the time for this event to occur.

2. Assessment of the dose-dependent inhibition of temperature-induced protein aggregation by molecular chaperones

Molecular chaperones are proteins that possess the unique ability to stabilise and prevent the aggregation of partially unfolded proteins. Here, we illustrate how a BMG LABTECH microplate reader can be used to assess the suppressive effects of molecular chaperones on the aggregation of "Protein X". As "Protein X" is heat labile, we have chosen to take advantage of the heating capabilities of the microplate reader by subjecting the protein to 60°C, in the presence or absence of the chaperone of our choice.

Procedure:

i) Prepare solutions of "Protein X" alone, chaperone alone, or "Protein X" with different concentrations of chaperone and then add them into separate wells of a microplate.

ii) Insert the plate into the pre-heated (60°C) BMG LABTECH microplate reader and adjust the instrument settings as shown:

- Wavelength: 340 nm
- Meas. start time: 0 s
- Cycle time: 120 s
- Number of cycles: 70
- Shaking time: 60 s
- Shaking: orbital
- Temperature: 60°C

iii) Run time course

Rev. 12/2005
Keywords: **Aggregation, Chaperones, Inhibition dose response, Protein, Protein folding**

Results & Discussion

Figure 1 shows the plot of protein aggregation by wild-type "Protein X" and its variants. Out of the 6 proteins tested 3 did not exhibit any detectable tendencies to self-aggregate.

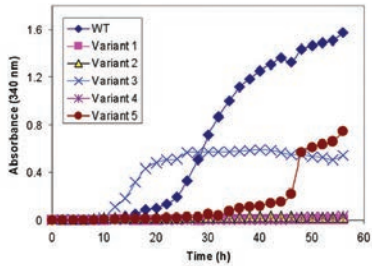

Fig. 1: Time course of protein aggregation by wild-type "Protein X" and its variants.

Thioflavin T binding studies showed high fluorescence associated with the presence of fibrous aggregates in 3 of the 6 samples; these were the same 3 samples that showed extensive aggregation by absorbance.

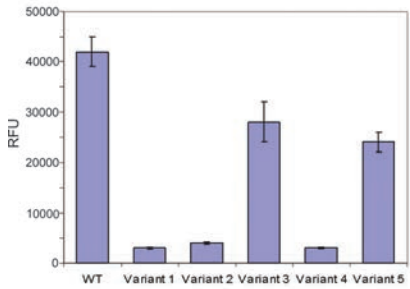

Fig. 2: End point fluorescence measurements associated with binding of fibril-specific Thioflavin-T.

We were able to incorporate these and other data obtained from activity tests to eventually show that variants 2 and 4 were good candidates for further therapy-related studies. The time course of heat-induced "Protein X" aggregation in the presence or absence of molecular chaperones is shown in figure 3.
Heating "Protein X" at 60°C resulted in extensive aggregation which reaches maximum by 20 min. In the presence of increasing amounts of the chaperone, the rate and extent of aggregation is significantly and proportionally reduced. These results clearly show that the molecular chaperone can inhibit the aggregation of "Protein X".

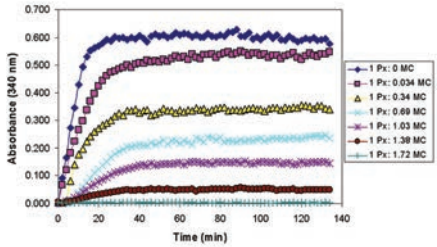

Fig. 3: Dose-dependent effects of the molecular chaperone (MC) on the heat-induced aggregation of "Protein X" (Px). Results shown are representative of 3 independent experiments.

Conclusion

We have demonstrated that the BMG LABTECH microplate reader is well-suited in medium to high throughput labs where screening of large sets of experimental conditions, is required.

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ADP Hunter™ assay for HTS of kinase inhibitors using the PHERAstar® *FS*

Lindy Kauffman, Uyen Hguyen
DiscoverX Corporation, USA

- **Generic screening of potentially all protein kinases in fluorescence mode using the PHERAstar® *FS***
- **Direct measurement of ADP and therefore cost effective: No need of specific antibodies or radioactive beads**
- **Simple and robust assay with Z' > 0.7**

Introduction

As mediators of eukaryotic signal transduction, controlling multiple cellular processes such as gene transcription, cell cycling, migration, apoptosis and differentiation, protein kinases are considered important targets in drug discovery. Kinase HTS screens ideally require kinase assay platforms with broad applicability in the inhibitor drug discovery process and are thus generically applicable to both the kinase target and substrate. Most non-radioactive kinase assay formats are limited in this respect in that they require either specific antibodies, or affinity capture reagents, to detect generation of the phosphorylated substrate. Often specific anti-bodies to the phosphorylated substrate are unavailable while affinity capture methods have limitations in the kinase assay conditions that can be used. During substrate phosphorylation, all kinases consume ATP and assays have been developed to measure ATP depletion occurring during the kinase reaction. However, this approach is limited by the ATP concentration required by the kinase, resulting in a high assay background. Consequently, small signal decreases, as occurs with weakly active kinases are difficult to detect.
An optimal approach is to measure the accumulation of a generic product of the kinase reaction i.e. ADP. This technique results in an increase in assay signal directly proportional to kinase activity and has the marked advantage that the assay is performed at a range of ATP concentrations, including those at the ATP K_m value. Consequently, the inhibitory potency of novel compounds, when evaluated at the ATP K_m, is a robust measure of activity at the ATP binding site and is easily compared to similar measurements at other kinases. Moreover, measurement of ADP accumulation allows flexibility in choice of the kinase substrate, so that both peptides and endogenous substrates (including during auto-phosphorylation) the kinase may be utilized.

DiscoverX has developed a homogeneuos fluorescence based assay to measure the generation of ADP, a universal product of kinase activity. The assay uses an enzyme-coupled reaction that produces a red-shifted fluorescence signal that is directly proportional to the amount of ADP in the solution. ADP Hunter is a biochemical assay to measure the accumulation of ADP (figure 1), a universal product of kinase enzyme activity.

ADP Hunter is specifically designed for high throughput screening of kinase inhibitors. The assay has been designed for use in full-volume 384-well microplates, but can also be run in additional microplate formats. To allow for automation, a Stop Solution is also provided for added signal and background stabilization. Unlike alternative generic approaches that monitor the depletion of ATP from a kinase reaction, this method follows the product of the reaction, and offers a convenient gain-of-signal assay format. This application note describes the use of the ADP Hunter assay measured on BMG LABTECH's PHERAstar *FS* multimode HTS plate reader.

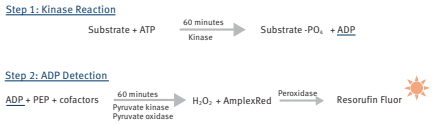

Fig. 1: Schematic principle of the ADP Hunter assay.

Materials & Methods

BMG LABTECH's PHERAstar *FS* combines rapid plate reading necessary for HTS with the enhanced performance and sensitivity needed to read small fluid volumes.

The PHERAstar *FS* is desinged to read all leading HTS detection modes such as fluorescence intensity, time-resolved fluorescence, fluorescence polarization, luminescence and absorption in all formats up to 1536. The PHERAstar *FS* was run in fluorescence mode for the monitoring of the ADP Hunter demo kit (DiscoverX Corporation) containing the following reagents:

Table 1: ADP Hunter demo kit components.

	Kit Components	Volumes
1	ADP Hunter Reagent A	8 mL
2	ADP Hunter Reagent B	15 mL
3	ADP Hunter Stop Solution	4 mL
4	ADP Hunter Standard (360 µM)	2 mL
5	ADP Hunter Assay Buffer	20 mL

The standard curve for DiscoverX's ADP Hunter kit was run according to the package insert protocol (table 2) in black walled 384-well plates (non-binding polypropylene plates; Greiner).

Table 2: ADP Hunter protocol for a standard curve.

Full Volume 384-well Plate	ADP Standard
Step 1: Standard Dilutions	8 µL ADP Standard dilutions
Step 2: ADP Detection	a) Add 8 µL Reagent A b) Add 16 µL Reagent B c) Incubate 60 minutes
Step 3: Stop Solution	Add 4 µL Stop Solution
Read fluorescence intensity signal: Resorufin excitation wavelength: 530-10 nm Resorufin emission wavelength: 590-20 nm	
Note: The signal may be measured up to 2 hours after addition of Stop Solution	

Rev. 05/2006
Keywords: **ADP, DiscoverX, Drug discovery, Inhibitor screening, Kinase**

The fluorescence signal (Ex: 530-10 nm/Em: 590-20) was read 1 hour after the addition of the last reagent on the PHERAstar *FS* using the following protocol for plate reader setup (figure 2).

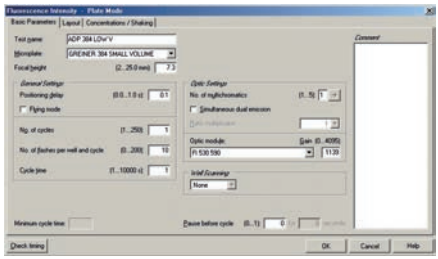

Fig. 2: ADP Hunter assay setup window from the PHERAstar *FS*, an optical module for the ADP Hunter is directly available from BMG LABTECH.

Results & Discussion

DiscoverX ADP Hunter assay was prepared in 384-well format and standard curves were run on BMG LABTECH's PHERAstar *FS* in fluorescence mode. ADP standard detection reagents were added according to the assay protocol and the fluorescence signal was read 1 hour after addition of the last reagent. The ADP standard curve is illustrated in the figure below (figure 3).

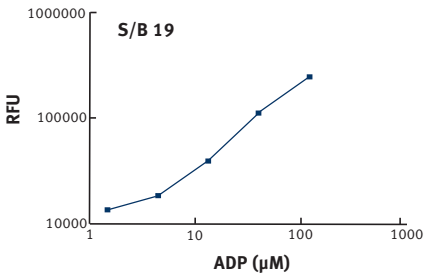

Fig. 3: ADP Hunter standard curve data run in 384-well format.

The ADP Hunter assay format was designed to produce a positive signal in direct proportion to the amount of ADP generated. The dynamic range of the assay is 1.5 µM to 120 µM ADP. The simple homogeneous assay format, provides robust and reproducible results with signal to background ratios > 10 and typical Z' values > 0.7. ADP Hunter is based on enzyme couples reaction that produces a positive red-shifted fluorescence signal, minimizing interference from fluorescent compounds. In addition, ADP Hunter incorporates a stop solution, which stabilizes the detection signal for screening plates in batch mode. Here we have described the value of a generic kinase screening assay when analyzed on a PHERAstar *FS* instrument. Together, they offer a powerful solution for kinase screening needs.

Conclusion

This assay is a useful tool for those customers who do not have access to a modified substrate, a phosphorylation-state specific antibody, or the ability or desire to use radioactivity. These reagents are designed to be robust and applicable to high throughput fluid dispensing systems using simple plate readers. The PHERAstar *FS* with its innovative and user friendly, intuitive software allows for the greatest flexibility and ease of use.

For more information on DiscoverX assays please refer to the web site: www.discoverx.com

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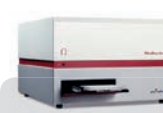

Omega Series

Analysis of prostate tumour cell invasion using BD FluoroBlok™ and a BMG LABTECH microplate reader

Claire A. Hart, Noel W. Clarke and Michael D. Brown
Paterson Institute for Cancer Research, University of Manchester, Manchester, UK

- **PC3-GFP cells were pre-labelled with SYTO 82, plated in BD FluoroBlok™ invasion chambers**
- **Real-time analysis of tumour cell invasion**
- **Comparable results from plates placed in a regular incubator and in a BMG LABTECH microplate reader**

Introduction

The mechanism of prostate metastasis to bone marrow is a complex multistage process that is only beginning to be understood. Initial steps include down regulation of molecular binding complexes and production of enzymes such as matrix metallo-proteinases leading to the loss of cell to cell adhesion within the tumour and extravasation of tumour cells. Once in the peripheral blood the circulating prostate tumour cells bind specifically to and invade through the bone marrow endothelial barrier and basement membrane, leading to formation of bone metastases.

Traditional invasion systems are time consuming with each assay involving washing, scraping with a cotton bud to remove non-invading cells, staining, drying and manual counting. Most importantly the assay is inflexible and limited in that the inserts can only present the researcher with a fixed end point. The BD FluoroBlok™ tumour cell invasion system is a high throughput alternative. The system is versatile and sensitive, involving no manipulation of inserts, thus reducing labour and time. It allows the researcher to do multiple real-time readings of the same inserts before and after introducing stimuli. The inserts are made of a dyed PET membrane that has a wide fluorescence blocking range from 490-700 nm allowing a range of potential fluorophores to be used. The system can be automated with the incorporation of the BMG LABTECH microplate reader, to increase efficiency and high throughput of assays for drug discovery, cell migration, motility and chemotaxis studies.

Principle

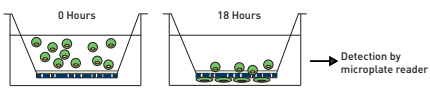

Fig. 1: In presence of an attractant cells migrate through the membrane of the inserts. They are no longer shielded from the light source and can be detected by the BMG LABTECH plate reader (reading from below).

Materials & Methods

All components of the tumour cell invasion system were purchased from BD Biosciences.

- HTS FluoroBlok™ Insert 24 well / 8 µM pores
- 24 well Companion Plate for use with 9mm pores
- BD Matrigel™ Matrix 5ml
- BD BioCoat™ Tumour Cell Invasion System 24-Multiwell Insert Plate 8 µM pre coated Matrigel™
- BMG LABTECH microplate reader with fluorescence detection and either Gas Vent Connection or Atmospheric Control Unit (ACU) for gas control

All tissue culture reagents and SYTO 82 were purchased from Invitrogen.
The FluoroBlok™ tumour cell invasion system was prepared as shown in Figure 2. 0All cultures were set up using phenol red free RPMI 1640 media. FluoroBlok™ cell culture inserts were coated with 100 µl of Matrigel™ (diluted at 1:25 with RPMI 1640) and allowed to set for 2hr at 37°C (Matrigel™ pre-coated FluoroBlok™ inserts can be used).

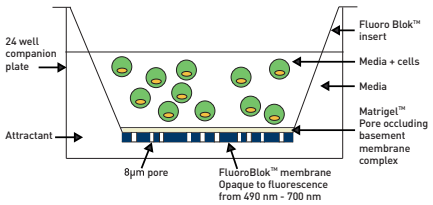

Fig. 2: Cell invasion set up.

Coated inserts were placed in the 24 well companion plate containing 1ml of RPMI 1640 / 0.1% BSA with or without chemoattractant. In the top half of the insert $2x10^5$ GFP or SYTO 82 labelled PC-3 cells were plated in 0.25 ml of RPMI 1640 / 0.1 % BSA. Plates were then incubated at 37°C for 18 hr to allow invasion to occur after which a final end point reading was taken on the BMG LABTECH plate reader. For real time invasion, the plate was placed in the BMG LABTECH reader pre warmed to 37°C and 5% CO_2 was inflated either with the ACU or via Gas Vent. The BMG LABTECH reader was set to take readings every hour for 21 hr.

Results & Discussion

Initially a comparison was made between the manual counting and the automated counting system of the FluoroBlok™ invasion chambers. Two identical assays were set up and counted manually or by the BMG LABTECH microplate reader. Both systems gave similar results (not shown).

Rev. 12/2006
Keywords: **Bottom reading, Fluoroblok, GFP, Invasion, Migration, Syto82**

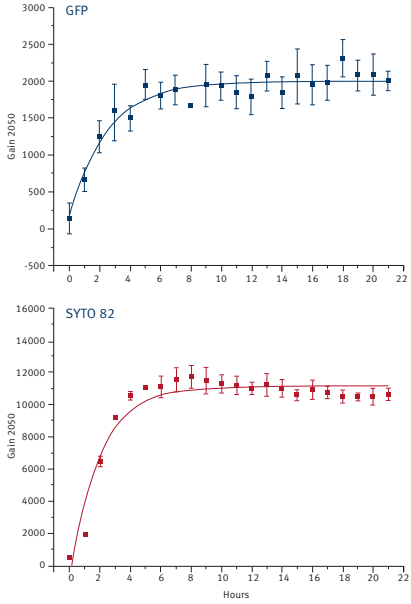

Fig. 3: PC3-GFP cells were pre-labelled with SYTO 82, plated in FluoroBlok™ invasion chambers and placed in the BMG LABTECH reader with either ACU or Gas Vent set at 37°C and left overnight inflated with 5% CO_2 air. Readings were taken every hour on both GFP and SYTO 82 wave lengths for a period of 21 hr.

Initially a comparison was made between the manual counting and the automated counting system of the FluoroBlok™ invasion chambers. Two identical assays were set up and counted manually or by the BMG LABTECH microplate reader. Both systems gave similar results (not shown).

Cells expressing a fluorescent protein are the ideal for use in these applications as no further manipulation is needed to be able to receive a signal when reading in the BMG LABTECH microplate reader. However, not all cells can be tranfected easily, especially primary cells. There are a number of alternatives to overcome this by either pre-labelling or post-labelling cells. Earlier investigations demonstrated that post-labelling was too unreliable hence pre-labelling was the method of choice. The problems inherent with prelabelling were the risk that the dyes could affect cell function and invasion. For this reason; the need for optimization of labelling time and side effects needed to be established beforehand. Pre-labelling is advantageous because it allows real-time invasion studies. The first step was to test whether the BMG LABTECH plate reader was able to support cells over 21 hours to gain real-time invasion data. PC3-GFP cells were pre-labelled with SYTO 82 and plated in FluoroBlok™ invasion chambers. These were placed in the BMG LABTECH reader with either ACU or Gas Vent set at 37°C overnight inflated with 5% CO_2 in air and set to take readings every hour of both GFP (488/520 nm) and SYTO 82 (530/570nm) for 21 hr. Figures 3 and 4 show that the BMG LABTECH reader supports the FluoroBlok™ invasion chamber assays over a period of 21 hr; showing similar data to an experiment where invasion chambers were left in an incubator (37°C / 5% CO_2 in air) overnight and read at specific times.

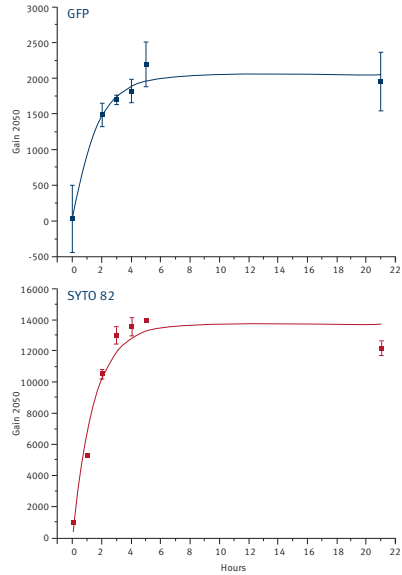

Fig. 4: PC3-GFP cells were pre-labelled with SYTO 82, plated in FluoroBlok™ invasion chambers and placed in a regular incubator overnight. Readings were taken on the BMG LABTECH reader every hour for the first 5 hr then a final reading at 21 hr. Plates were placed back in the incubator in between readings.

Conclusion

Using a BMG LABTECH microplate reader with integrated Gas Vent or Atmospheric Control Unit (ACU) for reading the FluoroBlok™ tumour cell invasion system gives results comparable to the traditional invasion assay. Compared with traditional invasion systems involving washing, staining and drying steps the introduced method is fast, saving time and labour.

BMG LABTECH readers are able to take multiple readings over time at a user defined temperature and the Gas Vent or ACU enables an environment conducive to cell proliferation and migration.

Size-selective assessment of tight junction paracellular permeability using fluorescently labelled dextrans

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- **Tight junctions encircle epithelial and endothelial cells**
- **Tight junctions restrict paracellular diffusion of ions and non-ionic tracers depending on charge and size**
- **Paracellular permeability of 4 kDa FITC Dextran and 70 kDa Rhodamine B Dextran is determined**

Introduction

Epithelial and endothelial cells are joined to each other via a set of intercellular junctions that are important for tissue formation and function. Tight junctions are composed of transmembrane components that mediate adhesion and form the paracellular diffusion barrier. These proteins interact with a cytoplasmic plaque composed of adaptor proteins that recruit various signalling components and interact with the actin cytoskeleton. Epithelial tight junctions create a functional boundary between the apical and basolateral cell surface domains, thereby regulating diffusion along the paracellular pathway. They also form a semi-permeable paracellular gate that restricts diffusion in a charge- and size-selective manner. The ion- and size-selectivity of the paracellular pathway differs among epithelia and is regulated by different physiological and pathological stimuli. Since the molecular mechanisms of size-selective diffusion are poorly understood, studing barrier formation by endothelia and epithelia is important, especially in treating such diseases as chronic inflammations or cancer. Here we describe a scalable, easy to use, and reliable tight junction permeability assay that analyzes tight junction integrity, as well as the rates of size-selective paracellular permeability.

Materials & Methods

- MCF-10A-95 cells and MCF-10-JB cells were cultured as described
- 12-well clusters of tissue culture inserts in polystyrene plates were from Corning. Culture systems from 6 to 96 wells are commercially available.
- Fluorescently labelled dextran and rhodamine-labelled dextran were from Sigma
- EVOM voltmeter, World Precision Instruments, Sarasota, Fl., USA for TER (transepithelial electrical resistance) measurements
- BMG LABTECH microplate reader for fluorescence measurements

General considerations

Many of the commonly used methods to analyze the tight junctions are based on cultured epithelial cells since they easily allow quantification of junctional properties. Furthermore, studies are generally performed with epithelial cells cultured on permeable supports since they allow easy measurements of electric currents or tracer flux across monolayers. Epithelial cells grown on such permeable supports often polarize and differentiate more extensively.

Investigation of tight junctions often involve transfection of wild type or mutant tight junction proteins; therefore, it is important that such cell lines express the transfected proteins in a homogenous manner. Furthermore, when a new cell line is to be used, pilot studies with non-transfected cells should be done to determine how quickly the cell line forms mature monolayers that exhibit stable functional junctions.

Analysis of the paracellular gate

Tight junctions restrict paracellular diffusion of ions and hydrophilic non-ionic tracers in a selective manner, differentiating by charge and size. The selectivity of the paracellular barrier varies from one epithelial tissue to another and different stimuli or manipulations can result in opposite effects on the permeability of ions and tracers. Ion permeability of tight junctions is generally determined by measuring transepithelial electrical resistance (TER). Paracellular permeability of hydrophilic tracers can be monitored with compounds that are labelled fluorescently, such as dextrans. Herein, fluorescently labelled dextrans (4kD FITC dextran and 70 kD Rhodamine dextrans) are used as tracers since different sizes can be analysed by the same detection method; moreover, the use of both FITC and Rhodamine labelled dextrans allows for the analysis of two different tracers in the same culture.

Experiments

Cells are plated to confluence in 12-well tissue culture inserts and are left for at least 5 days to form differentiated monolayers, with the medium being replaced every second day. The analysis is started by measuring TER for later comparison with the paracellular diffusion results. The medium is then replaced with fresh tissue culture medium, adding 1 ml to the outer and 250 µl to the inner chamber of 12-well tissue culture inserts. The cultures are then left to equilibrate in the tissue culture incubator for at least 30 minutes. Then 50 µl of the fluorescent tracer(s) is added into the inner chamber, bringing the total volume to 300 µl, so that the final concentration of tracer(s) is between 1 to 3 mg/ml. The cultures are then incubated at 37°C for 3 to 4 hours.
The filters are removed and the diffused fluorescent tracer is measured by the BMG LABTECH microplate reader (FITC-Dextran: Exc: 485 nm and Em: 544 nm and/or Rhodamine B-Dextran: Exc: 520 nm and Em: 590 nm). The amount of diffused dextran can be determined using calibration curves established just with the stock solution, and kinetic experiments can be performed by removing small samples of the outer chamber medium every hour.

Rev. 01/2007
Keywords: **Dextrans, FITC, Permeability, Rhodamine, Tight junction**

Results & Discussion

Neither TER nor tracer permeability measurements alone reflect the paracellular pathway properties, but both are a composite of the paracellular and transcellular route. For example, an increase in the 4kD-dextran permeability could be due to either increased paracellular diffusion and/or increased rates of fluid-phase transcytosis. That means that transcytosis needs to be considered. Figure 1 shows the results of TER measurements from two different cell strains, indicating that only MCF10A-95 cells form electrically tight monolayers.

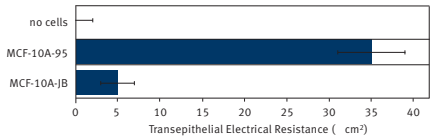

Fig. 1: Analysis of the paracellular gate in two different strains of MCF-10A cells. MCF-10A-95 and MCF-10-JB cells were cultured on permeable tissue culture supports for 5 days and then analysed by measuring transepithelial electrical resistance.

Paracellular permeability of 4kD FITC Dextran and 70 kD Rhodamine Dextran was determined by adding a mix of the two tracers to the apical chambers of the cultures. Figure 2 shows that both cell lines reduced the amount of paracellular flux of both types of dextran.

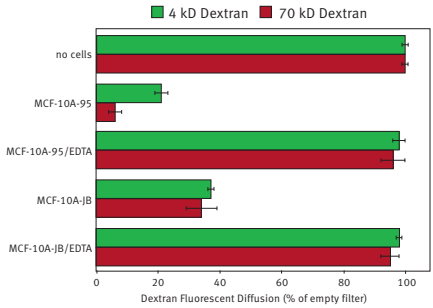

Fig. 2: Paracellular permeability of cultures incubated with dextran in the absence or presence of EDTA, which dissociates cell-cell junctions, was compared with diffusion across empty filters. Note, only the MCF-10A-95 strain develops a electrically resistant barrier that restricts paracellular flux by size.

This was calcium-dependent, suggesting that the formation of cell-cell junctions is important for barrier formation. The data shows that MCF-10A-95 cells not only form more efficient barriers than MCF-10A-JB, but they also restrict diffusion in a size-selective manner, supporting the conclusion that they form functional tight junctions. Thus, MCF10A-95 cells are able to form a functional epithelial barrier that restricts paracellular diffusion in an ion- as well as size-selective manner. These results further illustrate that assessment of the paracellular barrier formation requires the analysis of both TER and paracellular tracer diffusion.

Conclusion

When analyzing tight junctions, it is required to test for expression/ localization of protein markers as well as to assay for paracellular gate function. The junctional paracellular gate exhibits complex features; hence, a single assay does not allow a meaningful conclusion. Minimal analysis requires the determination of ion selectivity (TER) and size-selective tracer diffusion.

We describe here a method for the quick and reliable analysis of size-selective paracellular tracer diffusion using fluorescent dextrans of different molecular weights. The assay is robust and allows for the analysis of many different samples in parallel. The assay could easily adapted to large scale screens to identify genome-wide regulatory pathways or small molecules to modify junctional permeability.

Using the BMG LABTECH microplate reader for these experiments allows quick and consistent fluorescence measurements in either kinetic or endpoint format.

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Internalisation of fluorescently labelled phagocytosis targets quantified by a BMG LABTECH microplate reader

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- **Fluorescently labelled polystyrene beads were used as targets for phagocytosis studies**
- **Quantification of internalisation was done using the BMG LABTECH microplate reader**
- **Macrophages derived from blood monocytes were used as a model to examine alveolar macrophage function**

Introduction

Phagocytosis forms a major part of the innate immune system of higher eukaryotes. It involves the ingestion and destruction of potentially pathogenic or inflammatory particles, such as microorganisms, apoptosing cells and minerals. In humans, phagocytosis is initiated by the recognition of foreign particles, which can occur via one of two mechanisms. The first involves the direct recognition of repeated patterns of molecular structures on the surface of the microorganisms, such as the Gram-negative bacterial coat protein, lipopolysaccharide (LPS), and heat shock protein-60 (HSP-60).

Alternatively, pathogens can be detected indirectly by the recognition of humoral components from serum, that bind to the surface of pathogens, a process known as opsonisation. The opsonising proteins involved include antibodies and complement proteins which are recognised by Fc and C3b or C3bi receptors respectively. Following recognition, the target is internalised into a phagosome within the cell. This then fuses with a lysosome, which then releases proteases that catalyse the breakdown of the particle.

Alveolar macrophage (AM) phagocytosis of particles and microorganisms is an essential lung host-defence mechanism. In chronic lung diseases, such as chronic obstructive pulmonary disease (COPD), all pathophysiological changes are thought to be mediated by the macrophage. However, studying the process of pathogen clearance in the lung is difficult because obtaining viable AM from bronchoalveolar lavage (BAL) is variable. In contrast, blood cells are easily obtained, therefore a blood macrophage model can be used to examine AM functions. Macrophages are derived from monocytes, which can be separated from peripheral blood mononuclear cells (PBMCs). The differentiation of a monocyte to a macrophage takes place over 7-21 days.

Measurement of phagocytosis has traditionally involved microscopy, flow cytometric analysis or radioactive assays; all of which are time consuming, and in the case of the latter, require additional safety procedures. In this study, the use of a BMG LABTECH microplate reader for quantifying the internalisation of fluorescently labelled phagocytosis targets by monocyte derived macrophages (MDM) was investigated.

The BMG LABTECH readers are able to take multiple readings over time at a user defined temperature and can also be equipped with smart dispensing injection.

Materials & Methods

- BMG LABTECH microplate reader
- Black 96-well clear bottomed culture and assay plates (FisherScientific)
- Fluorescently labelled polystyrene beads (2 µm, carboxylate modified yellow-green, Invitrogen)
- Discontinuous Percoll gradients (Amersham Biosciences)
- Monocyte Isolation Kit II (Miltenyi Biotec)
- Granulocyte macrophage colony stimulating factor (GM-CSF, Biosource)
- Trypan blue (Sigma-Aldrich)

Monocytes were isolated from whole blood obtained from nonsmokers, using dextran sedimentation, discontinuous Percoll gradients and Monocyte Isolation Kit II. These were cultured on black 96-well clear bottomed culture plates with 2ng/ml GM-CSF for 12 days. After this time the monocytes had differentiated to a macrophage-like phenotype.
Alveolar macrophages (AM) were isolated from non-smoking patients undergoing investigative bronchoscopy. Lungs were lavaged with warm saline, which was then retrieved using a fibreoptic bronchoscope. Cells were washed and resuspended in media, then cultured on black 96-well clear bottomed plates. Both kind of cells, MDM and AM, are strongly adherent.
Phagocytosis assays were carried out using fluorescently labelled polystyrene beads (2µm, $50x10^6$ beads/ml). Macrophages (MDM or AM) were incubated with beads for 4h then washed to remove unbound particles. Extracellular fluorescence was then quenched using Trypan blue. The fluorescence of internalised beads was measured on the BMG LABTECH microplate reader with excitation at 480 nm and emission at 520 nm. To confirm internalisation of particles, cells were also viewed using a confocal microscope. DAPI was used to stain nuclei, and Evans blue for cytoplasm.

Results & Discussion

In order to establish the optimal incubation time for phagocytosis assays, MDM were isolated incubated for 2, 4 and 6h with fluorescent polystyrene beads. Fluorescence of ingested beads was quantified using a BMG LABTECH microplate reader. These experiments demonstrate that MDM can phagocytose fluorescently labelled polystyrene beads in a time dependent manner (Fig. 1).

Rev. 05/2007
Keywords: **Blood, Internalisation, Macrophages, Monocytes, Phagocytosis**

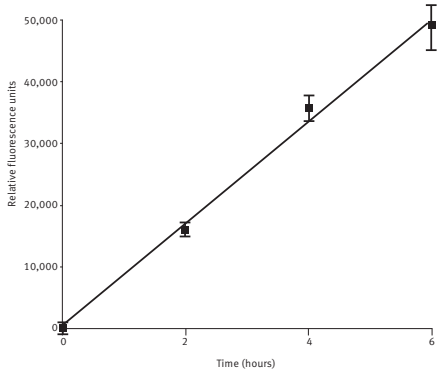

Fig. 1: Effect of incubation time on phagocytosis of polystyrene beads by MDM. MDM were incubated with 100µl fluorescent beads (50x10⁶beads/ml) for the times indicated. Fluorescence of internalised beads was measured on a BMG LABTECH microplate reader. Data are presented as mean±SEM, n=6.

In order to ascertain the optimal concentration of beads to be used in phagocytosis assays, MDM were incubated with solutions of between 0.5-50x10⁶ beads/ml. Phagocytosis of fluorescently labelled polystyrene beads increased in a concentration dependent manner (Fig. 2).

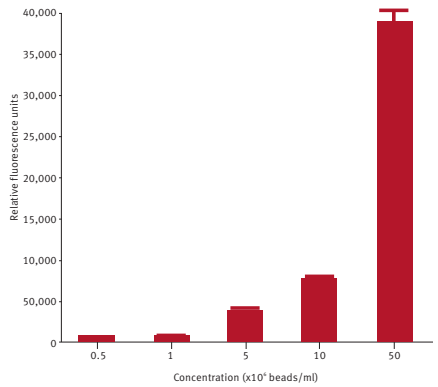

Fig. 2: Effect of concentration of polystyrene beads on phagocytosis by MDM. MDM were incubated for 4 h with beads solutions at concentrations indicated. Fluorescence of internalised beads was then quantified using a BMG LABTECH microplate reader. Data are presented as mean±SEM, n=7.

In order to confirm that beads were being internalised and not adhering to cell surfaces, MDM were cultured on chamber slides and incubated with polystyrene beads at increasing concentrations. These slides were then viewed using a confocal microscope. Images taken sequentially through the cells showed the beads to be completely within the cell cytoplasm (images not shown). Additionally, the images verified the dose dependent increase in phagocytosis with concentration of beads. To ensure that MDM phagocytosis reflected that seen in lung derived macrophages, comparative experiments using alveolar macrophages (AM) were performed. MDM and AM were incubated with beads and phagocytosis was measured using a BMG LABTECH microplate reader (figure 3).

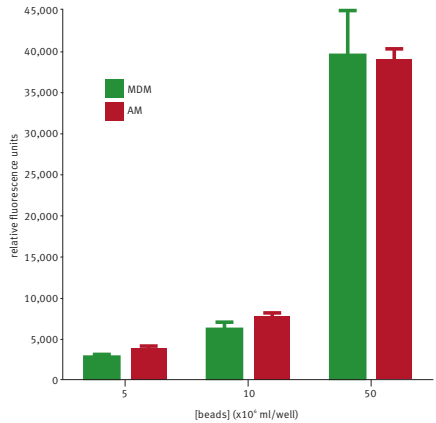

Fig. 3: Comparison of phagocytosis of polystyrene beads by MDM and AM. MDM and AM were incubated for 4h with fluorescently labelled beads at concentrations indicated. Phagocytosis was then quantified by measuring the fluorescence on a BMG LABTECH microplate reader. AM n=4, MDM n=7.

Conclusion

The findings presented here suggest that MDM can phagocytose polystyrene beads in a comparable manner to alveolar macrophages, and therefore are a suitable model to study phagocytosis. The BMG LABTECH microplate reader provides an accurate, consistent method for quantifying macrophage phagocytosis that is significantly more time efficient than previously employed methods.

High-throughput method for dynamic measurements of cellular viability using a BMG LABTECH microplate reader

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- **Cell viability was measured using the propidium iodide (PI) assay**
- **Kinetic measurements over 24 h allow for determination of lag times and death rates**
- **The BMG LABTECH microplate reader was used to record PI fluorescence over long periods**

Introduction

An accurate and reliable method of assessing cellular viability is a vital requirement for any work related to cell culture. This includes drug toxicity studies in addition to basic monitoring of the longterm stability of a culture. Currently there is a wide range of assays that can be used to assess cellular viability based on various different biochemical and molecular principles.

In general, there are two main ways in which a cell can be defined as non-viable. If the cell has: (1) a compromised plasma membrane or, (2) has lost its metabolic functioning. Dyes such as trypan blue and propidium iodide are non-permeable to the plasma membrane.

Therefore, only cells with a compromised plasma membrane will take up these types of dye. Another way of identifying a compromised plasma membrane is to detect substances in the extracellular medium which are normally only found within the cytosol of a cell. Therefore they must have leaked through the plasma membrane in order to reach the outside of the cell. An example of this type of method is the lactate dehydrogenase assay, which detects the cytosolic enzyme (lactate dehydrogenase) in the extracellular medium.

The work described in this application note is based on the propidium iodide (PI) assay. This is a very powerful assay, which allows viability to be measured dynamically over long periods of time. This is very useful when studying the effects of various substances, which may not necessarily have an instantly induced effect on the cell. Therefore this method enables the identification of any delayed death, which may be missed when only assessing viability at one specific timepoint. Furthermore, since the measurement is dynamic over a long period of time (24 hrs), the rate at which the death is occurring can be fully investigated.In this experiment we describe a further development of a high throughput method for dynamically assessing neuronal death with the help of a BMG LABTECH microplate reader.

Materials & Methods

- Black 24-well plates (Iwaki, Scientific Laboratory Supplies LTD)
- Propidium iodide (PI, Sigma)
- BMG LABTECH microplate reader

Cerebellar granule cells were obtained from post-natal Wistar rats (P5-8) and cultured directly onto 24-well plates at a plating density of 280-300,000 cells per well. PI was diluted with dH_2O to a concentration of 5 mg/mL and used at a final concentration of 50 µg/mL (diluted in experimental buffer referred to as 'Ctrl') (Protect the PI from light since it is photosensitive).
The BMG LABTECH microplate reader was set to fluorescent mode with an excitation wavelength of 544 nm and an emission wavelength of 612 nm. The culture medium from each well was removed and replaced with 500 µL of the PI-Ctrl solution. Four measurements were made at 60-second intervals in order to establish a resting level of PI fluorescence (pre-stimulation). This PI-Ctrl solution was removed and stored in a separate 24-well plate. The cultures were incubated for 60 minutes at 27°C in various concentrations of glutamate all diluted in an experimental buffer containing 50 µg/mL PI, 10 µM Glycine (Sigma, UK) and no Mg^{2+}. Throughout this 60-minute incubation period, measurements were taken at 15-minute intervals. In order to obtain a background level for the PI solution, the PI-Ctrl solution was measured. The glutamate-PI solution was then removed and replaced with the PI-Ctrl solution. Measurements were taken at 30-minute intervals for 23 hours. At the end of the 24 hours, the PI-Ctrl solution was removed and either EtOH (100 %) or Triton X (0.02 %) was added to each well in order to induce maximal death. Then the PI-Ctrl solution was added back and 5 measurements of this level of maximal PI fluorescence were taken.

Results & Discussion

The signal curves in Fig. 1 represent the changes in PI fluorescence depending on the glutamate concentration (0-200 µM). With increasing concentration of glutamate the resulting PI signal is also significantly increased.

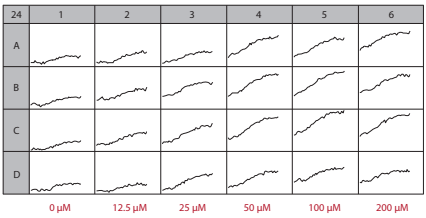


Fig. 1: Current State Picture of PI fluorescence measurement by various concentration of glutamate.

For quantifying the changes in the PI signal two methods can be used: (1) difference of each individual PI signal from its corresponding resting level (Figure 3 and 2) the

Rev. 04/2008
Keywords: **Cell death, Cell viability, Inhibition dose response, Long-time kinetic, Propidium iodide**

percentage death normalised against the resting level (0%) and the maximal death (100 %) induced by EtOH (100 %) or Triton X (0.02 %) (Figure 4) referred to as the "Min-Max range". Both methods show that the death induced by the glutamate is dose dependent. Furthermore, using the temporal dimensions of the measurements, one can estimate the time it takes for a significant amount of death to appear (initiated). In these experiments, the lag period between the end of the glutamate exposure and the initiation of significant levels of death is dependent on the glutamate concentration hence, cultures exposed to larger doses of glutamate have shorter lag periods.

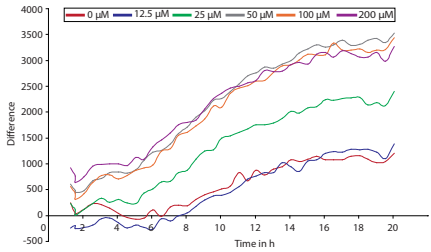


Fig. 2: Dose-response curve for the excitotoxic effects of glutamate. Propidium iodide signals are expressed as a difference between each individual PI measurement and its original resting level. The data was averaged from 4 replicates for each glutamate concentration.

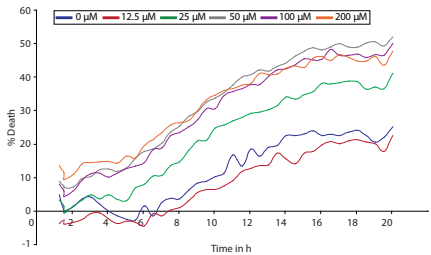
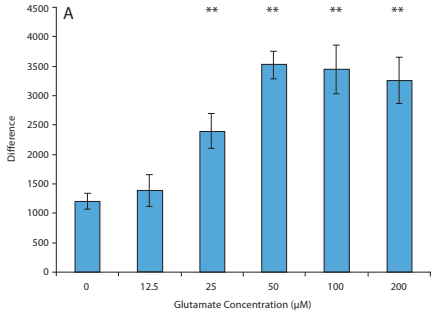


Fig. 3: Dose-response curve for the excitotoxic effects of glutamate. Propidium iodide signals are expressed as a percentage death which is normalized against the resting level (0%) and the maximum death level induced by EtOH/Triton x (100 %). The data was averaged from 4 replicates for each glutamate concentration.



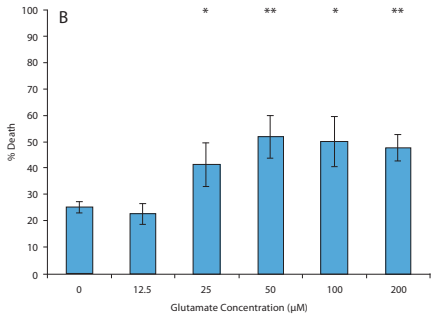


Fig. 4: Dose-response curve for the excitotoxic effects of glutamate. (A) Shows the maximum PI signal (Pre-EtOH/Triton treatment) as a difference ±S.E.M (Unpaired Student 1-tailed T-test, where *p<0.05, **p<0.01), as described in legend to figure 3. (B) Shows the maximum PI signal (Pre-EtOH/Triton treatment) as a percentage death ±S.E.M (Unpaired Student 1-tailed T-test, where *p<0.05, **p<0.01), as described in legend to figure 3.

Conclusion

We show that the PI assay is a very useful tool for monitoring neuronal viability. It allows a complete analysis of the dynamics of the response to a specific substance, which in our case is the neurotransmitter glutamate.
Numerous parameters, including lag times and rates of death can be assessed using this PI method. This is advantageous compared to other methods used for assessing neuronal viability since most of these methods usually involve a single end-point measurement or require termination of the preparation.

Investigation of the stereoselectivity of an anti-amino acid antibody utilizing tryptophan fluorescence

Danielle M. Corgliano, Heike Hofstetter, and Oliver Hofstetter
Northern Illinois University, DeKalb, IL, USA

- **Change in intrinsic tryptophan fluorescence is used to measure the binding of an antibody to its antigen**
- **Stereoselective antibodies specifically bind to the D-enantiomer of amino acids**
- **Effect of pH, temperature, and concentration on intrinsic tryptophan fluorescence**

Introduction

The binding sites of proteins such as antibodies are known to often contain tryptophan (Trp) residues, whose fluorescent properties may be altered upon ligand binding. Conformational changes within the binding site or simply the presence of the ligand can result in either fluorescence quenching or enhancement, which may be utilized to quantitatively investigate protein-ligand interactions. Highly stereoselective antibodies to amino acids have been used in a variety of analytical techniques for the sensitive detection of enantiomeric impurities and forenantiomer separation. The objective of this study was to test if tryptophan fluorescence can be used to determine the affinity of an anti-D-amino acid antibody toward a variety of standard and non-standard amino acids.

In order to examine the utility of BMG LABTECH microplate readers for measuring Trp fluorescence (Figure 1), experimental conditions were first optimized using the free amino acid as analyte.

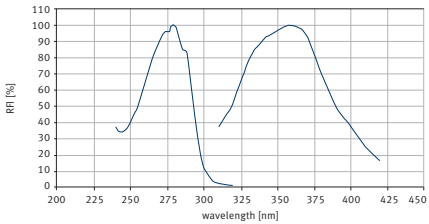


Fig. 1: Excitation and emission spectra of Tryptophan (dissolved in 50 mM PBS buffer, pH 7.4) performed on a BMG LABTECH microplate reader.

Materials & Methods

- BMG LABTECH microplate reader
- Reacti-Bind White Opaque 96-well plates (PIERCE)
- D,L-Tryptophan (Sigma)
- D-Phenylalanine (Sigma), L -Phenylalanine (Sigma)
- Phosphate buffers (pH values between 2 and 12)
- Monoclonal anti-D-amino acid antibody

96-well microtiter plates were blocked with 1% gelatin in PBS/0.05% Tween 20 (250 µL/well; 2 h at 37°C), followed by washing with PBS/0.05% Tween 20. Samples containing either Trp (200 µL/well) or antibody at varying concentrations in phosphate buffer (100 µL/ well) were excited at 280 nm; emission was detected at 350 nm. For ligand binding studies, 50 µL/well of antibody at a fixed concentration (30 µg/mL) was pre-incubated for 2 hours at RT with ligand in varying concentrations (50 µL/well) before fluorescence measurement.

Results & Discussion

The effect of analyte concentration, temperature, and pH was investigated in order to establish optimal conditions to be used with BMG LABTECH microplate readers. As seen in Fig. 2, a clear concentration-dependent increase in Trp fluorescence was observed at concentrations ranging between about 1 mM and 1 µM. Excellent signal-to-noise ratios with minimal background fluorescence were obtained upon excitation at 280 nm and detection of fluorescence emission at 350 nm. In contrast, no fluorescence was observed using phenylalanine in the same range of concentrations (not shown).

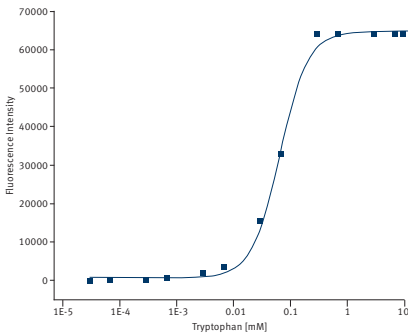


Fig. 2: Fluorescence intensities obtained with Trp at varying concentrations in phosphate buffer, pH 7.4.

The effect of temperature and pH was investigated using Trp at a concentration of 70 µM as analyte. As seen in Fig. 3, fluorescence intensity significantly decreases at higher temperatures.

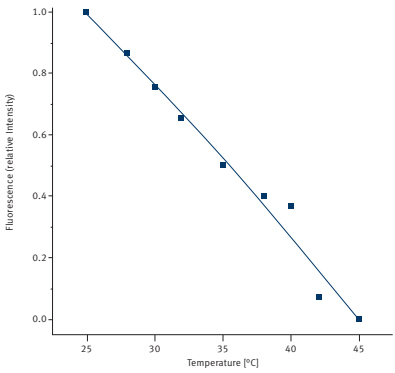


Fig. 3: Effect of temperature on Trp fluorescence.

Rev. 11/2007
Keywords: **Antibody, Protein-ligand interaction, Stereoselectivity, Tryptophan**

Also changes in the pH have a considerable effect on Trp fluorescence, which is strongest at a pH around 11 (Fig. 4). Both results are in good agreement with previous reports.

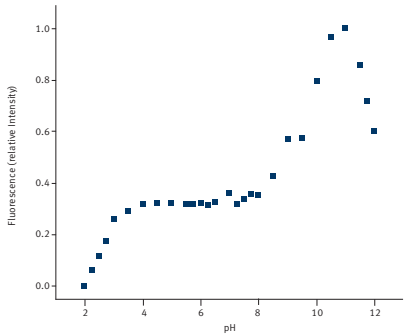


Fig. 4: Effect of pH on Trp fluorescence.

Investigating antibody stereoselectivity by measuring Trp fluorescence

As seen in Fig. 5, the BMG LABTECH plate reader can be used to determine the intrinsic Trp fluorescence of the anti-D-amino acid antibody used in this study. Increasing concentrations of the antibody in phosphate buffer, pH 7.4, resulted in increasing fluorescence emission at 350 nm upon excitation at 280 nm.

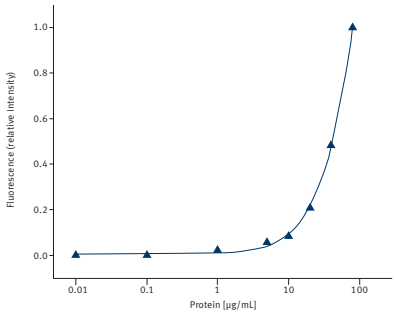


Fig. 5: Intrinsic Trp fluorescence of an anti-D-amino acid antibody at varying concentrations.

For protein-ligand studies, a fixed concentration of the antibody was incubated with the D- or L-enantiomers of a variety of amino acids. Fig. 6 shows the results obtained with D- and L-phenylalanine, respectively.

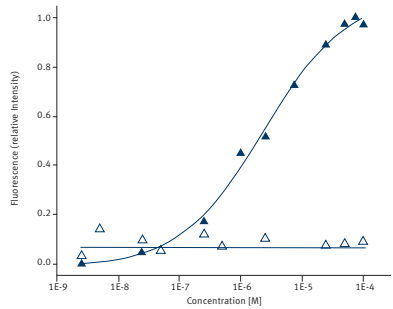


Fig. 6: Binding of D-phenylalanine (filled triangles) to an anti-D-amino acid antibody causes a concentration-dependent enhancement of the protein's intrinsic Trp fluorescence, while no such effect is observed using L-phenylalanine (open triangles) in varying concentrations.

While the interaction of the antibody with D-Phe causes a concentration-dependent increase of the antibody's intrinsic Trp fluorescence, no such effect is observed using the L-enantiomer. Similar results were obtained with the enantiomers of cyclohexylalanine, histidine, norleucine, leucine, and norvaline (not shown). In all cases, the stereoselective interaction of the antibody with the D-enantiomers of these amino acids caused a concentration-dependant enhancement of the protein's intrinsic Trp fluorescence, while no change in fluorescence was caused by the L-enantiomers. As observed using other analytical techniques, the affinity of the antibody is strongest to D-amino acids having aromatic or bulky side chains, while aliphatic amino acids are bound more weakly.

Conclusion

The BMG LABTECH microplate reader allows measurement of Trp fluorescence with high sensitivity and good signal-to-noise ratios. Excitation of appropriate proteins at 280 nm and measurement of fluorescence emission at 350 nm can be employed to investigate protein-ligand interactions and to deduce, e.g., binding affinities.

Use of BMG LABTECH microplate reader to monitor amyloid formation

Sarah Shammas and Ann-Christin Brorsson
Department of Chemistry and Cavendish Laboratory, University of Cambridge

- **Abberant protein aggregation implicated in several degenerative diseases**
- **Protein aggregation kinetic followed with a BMG LABTECH microplate reader**
- **Custom-made script allows for timed ex situ experiments**

Introduction

Amyloid fibrils are implicated in a number of diseases, known as amyloidosis, including type II diabetes and Alzheimer's disease. Each disease is associated with the misfolding of a particular protein into linear aggregates (fibrils) that accumulate in the body's organs as plaques. Various different species may be formed during the aggregation process, including linear precursors known as protofilaments, which often appear in the early stages. There is evidence that amyloid formation could be a generic property of the polypeptide chain i.e. during appropriate (partially destabilising) conditions, "any" protein will form amyloid fibrils. Despite this property it is clear that the amino acid side chains contribute to the stability and the morphology of amyloid aggregates, as well as the kinetics of their formation. In this study various mutants of an amyloid forming protein V have been designed to investigate the effect of the mutation on the aggregation behavior.

One group of fluorescent dyes which have been shown to bind to amyloid fibrils are the anilinonaphthalene sulphonic acid's (ANS). These dyes are not "amyloid-specific"; they bind to proteins via exposed hydrophobic patches on the surface. In this study the dye bis-ANS has been used to follow the aggregation process of protein V and mutants to give information about the kinetics and the hydrophobic properties of the formed amyloid aggregates.

In this report we demonstrate that the BMG LABTECH microplate readers can monitor amyloid formation by performing fluorescence kinetics studies. We have made particular use of the script mode feature, allowing us to use a simple procedure for making timed ex situ measurements. The model protein V that has been used in this study is associated with amyloid disease.

Materials & Methods

Aggregation kinetics monitored by bis-ANS fluorescence

To prevent any interference of the aggregation process by including dye in the reaction mixture, ex situ measurements were made. Aliquots of the aggregation mixture were removed at various time-points and added to a buffered solution of bis-ANS (obtained from Sigma- Aldrich for fluorescence measurements). This approach means that each well in the well-plate represents a single measurement in the kinetic assay. We have designed a script using BMG LABTECH's Script mode that allows us to accurately probe the fluorescence properties at ach time-point. The script runs a series of standard fluorescence protocols in a defined order allowing fluorescence measurements for consecutive wells. Additionally, it moves the wellplate in and out at appropriate times to assist sampling, and records the reaction time for each sample. By selecting the data to be exported to a CSV file, the results of the consecutive measurements (and the corresponding reaction times) can be recorded in a single file which can be opened and analyzed by a range of commercial and freeware programs.

Procedure

Buffer A: 10 µM bis-ANS in 50 mM sodium phosphate buffer pH 7.4

Buffer B: 50 mM sodium phosphate buffer pH 7.4

i. Protocols, one for each well measured, should be prepared. The protocols themselves are all identical apart from the well number. The instrument settings are as shown:
- Type: Fluorescence Slow kinetics
- Excitation filter wavelength: 400nm
- Emission filter wavelength: 500nm
- Temperature: 30°C
- No shaking

ii. Select CSV file format – also select the following options:
- append data to the end of file (rather than replacing previous data)
- output filename as <ID1>
- report style such as Short Header which outputs the relevant variables e.g. our script uses <ID2> to represent the reaction time

iii. Insert the plate into the BMG LABTECH microplate reader

iv. Open script

v. Edit script: choose a filename for the CSV output i.e. <ID1>

vi. Save and run script (all actions are prompted by script from now on)

vii. Pipette 45 µL of A and 5 µL of B into the first well as a control.

viii. First measurement is made.

ix. Pressing enter starts a countdown. After 30 s a beep sounds – this is a prompt to start the aggregation by adding buffer B to protein V to a protein concentration of 30 µM.

x. Pipette 45 µL of A and 5 µL of aggregation mixture into the next well

xi. Measurement is made

Repeat x-xi until sufficient data has been collected, and then stop script.

Final Bis-ANS fluorescence intensities for the different mutants Standard concentration curves were constructed. Various volumes (instead of 5 µl) of mature fibrils (30 µM) were added to 45 µl of buffer A. The concentration of bis-ANS was kept constant by addition of buffer B to a final mixture volume of 50 µl.

Rev. 01/2008
Keywords: **Aggregation, Alzheimer's, Amyloid formation, Bis-ANS, Fibril formation**

Results & Discussion

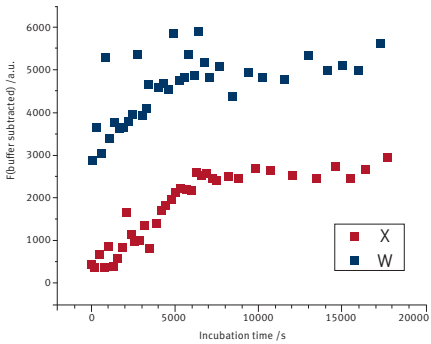


Fig. 1: Kinetics of aggregation of proteins X and W monitored by ex situ bis-ANS fluorescence.

Figure 1 shows the kinetic plot of the aggregation process for two mutants of protein V (X and W). At time = 0 s protein X has a very low fluorescence signal, this increases until it reaches a fairly stable level at around 7000 s. The increase likely represents conversion of the monomer into mature fibrils with hydrophobic surfaces. The fluorescence with protein W increases over the same time-scale as protein X, however the signal for protein W is consistently around 2500 a.u. higher than the signal for protein X. The non-zero initial fluorescence of protein W suggests the presence of aggregates, formed rapidly within the dead time of the experiment, that are able to bind bis-ANS. That these are not formed for protein X is interesting since it is generally thought that early oligomers represent the toxic species in amyloid diseases (rather than the fibrils). The higher fluorescence signal found for Protein W after stabilization of the bis-ANS signal suggests that the mature aggregated species formed from this mutant are also more hydrophobic than species formed from protein X. Differences in the hydrophobic nature between aggregated species are also interesting to study since a hydrophobic species could have the potential to mediate toxicity by interacting with the cell membrane in vivo.

The bis-ANS binding of protein V and another mutant Y was also investigated at longer times. Proteins were left under aggregating conditions for 3 weeks allowing fibril formation. Aliquots were then removed to create a standard concentration curve for each protein (see Figure 2).

Linear fits to the data were made and the gradients and R values for each protein are shown in Table 1. A good fit was obtained for protein V, however that for protein Y was poor despite a reasonably high fluorescence intensity. This may reflect a very heterogenous population of species (observed by TEM, data not shown). Protein Y also has a lower fluorescence signal than protein V, indicating that less hydrophobic surfaces are exposed.

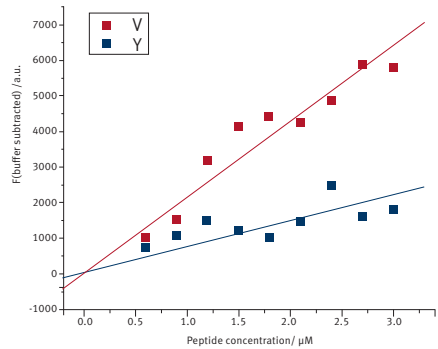


Fig. 2: Bis-ANS fluorescence intensity as a function of protein concentration (bis-ANS concentration is constant between samples) for the proteins V and Y.

Protein	Gradient	R
V	2135 ± 91	0.95
Y	740 ± 76	0.72

Table 1: Linear fits for each standard curve of fig. 2

Conclusion

In this report we have demonstrated that BMG LABTECH microplate readers can be used to follow protein aggregation kinetics using fluorescence measurements. In particular we have shown that using the script mode it is possible to make timed ex situ measurements fairly simply, creating an output into a single CSV file.

Molecular Probes® NanoOrange® assay performed on a BMG LABTECH microplate reader

Tracey Madgett
University of the West of England, Bristol; Faculty of Health and Life Sciences, Frenchay Campus, Coldharbour Lane, Bristol, BS16 1QY, UK

- **NanoOrange® Assay for protein quantitation performed on a BMG LABTECH microplate reader**
- **High and small concentration range evaluated**
- **Kit can easily be adapted for high throughput measurements**

Introduction

The field of proteomics has expanded dramatically in recent years with research on a whole host of organisms. Techniques such as two dimensional difference gel electrophoresis (2D DIGE) require the accurate quantitation of protein, as up to three labelled samples can be loaded on a single gel for comparison.
Assay methods for determining protein quantitation include absorbance at 280 nm, the Bradford assay, Lowry assay, BCA method and more sensitive assays such as Fluoroprofile® (Sigma-Aldrich) and NanoOrange® (Molecular Probes®).
The measurement of solution absorbance at 280 nm (A280) has problems with variability between samples and interference from nucleic acids and other contaminants. Detergents and reducing agents can cause problems with the other assays mentioned above – these agents are present in samples for 2D gel electrophoresis.

Fluorescent methods are more sensitive for quantitating proteins than absorbance methods. The NanoOrange® protein quantitation assay from Molecular Probes® is a highly sensitive assay with the useful range being between 100 ng/mL and 10 µg/mL for fluorescence based microplate readers. The spectrum of the NanoOrange® reagent is given in Figure 1.

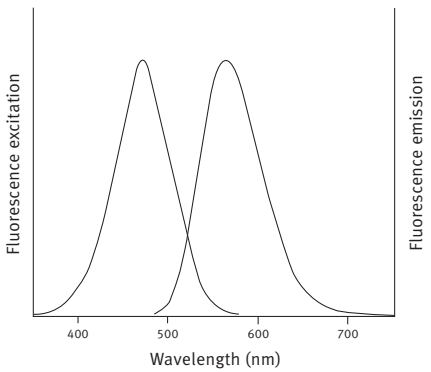


Fig. 1: Excitation and emission spectra of the NanoOrange® reagent. Copyright Invitrogen Corporation. Used with permission.

Materials & Methods

The following materials were supplied by the manufacturers as detailed:

- NanoOrange® Protein Quantitation Kit
- MJ Research PTC200 Peltier Thermal Cycler
- BMG LABTECH microplate reader
- Microplates, black 96 well (Greiner Bio-One)
- General laboratory consumables included pipette tips and microcentrifuge tubes

Reagents:

- NanoOrange® Protein Quantitation reagent A
- NanoOrange® Protein Quantitation diluent B
- Bovine Serum Albumin (BSA) Standard (2 mg/mL)

The working NanoOrange® reagent was made by diluting reagent A 500-fold in a 1:10 dilution of diluent B. A stock BSA solution at 10 µg/mL was made by diluting 1:200 in working NanoOrange® reagent. A serial dilution of the stock BSA solution was performed, yielding further concentrations ranging from 0.2 µg/mL to 10 µg/mL. The blank solution was working NanoOrange® reagent alone. Dilutions of samples were made in working NanoOrange® reagent (for example, 1:100, 1:500, 1:1000). Following denaturation of the standards and samples, 100 µL of each was placed into a microplate for measurement. The prepared 96-well plate was inserted into the instrument and read in fluorescence mode with the following parameters:

Excitation filter:	485-12
Emission filter:	570-10
Gain:	plate assessed, well with the highest intensity selected
Number of cycles:	1
Number of flashes per well:	10

The data was evaluated using the BMG LABTECH MARS data analysis software. The average value of the blank measurement was subtracted from the measurements and the standard curve was plotted.

Rev. 01/2008
Keywords: **A280, BSA, NanoOrange, Protein quantification**

Results & Discussion

A four parameter fit was performed on the data which yielded a very high R-value of 0.9998. (Figure 2).

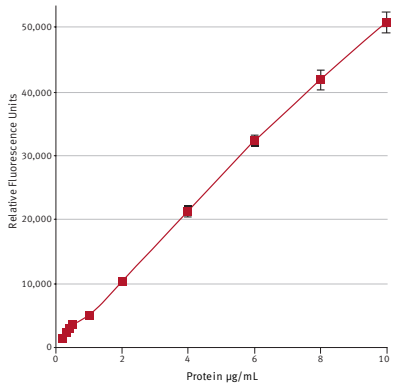


Fig. 2: BSA standard curve obtained with the NanoOrange® Protein Quantitation Kit. A BSA concentration range of 0.2-10 µg/mL is used. A gain of 1967 was optimized on the highest protein concentration.

The small concentration range was also measured separately with optimized gain on the highest protein concentration (Figure 3).

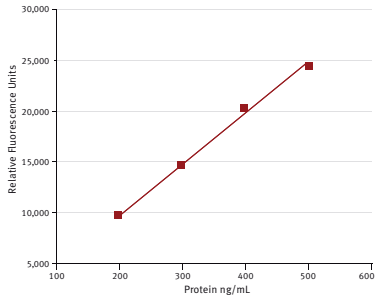


Fig. 3: Small concentration range BSA standard curve obtained with the NanoOrange® Protein Quantitation Kit. A gain of 2721 was optimized on the highest protein concentration (12 replicates).

A linear relationship between protein concentration and fluorescence units is obtained indicating that small concentrations of protein in samples can be determined using the BMG LABTECH microplate reader.

Conclusion

The NanoOrange® Protein Quantitation Kit can be easily adapted for use with a fluorescent microplate reader such as BMG LABTECH´s and used in a high throughput manner.

The instrument allows measurement of endpoint or slow and high kinetics at a user-defined temperature and can be easily equipped with injectors.

Measuring mitochondrial membrane potential using the FLUOstar® Omega microplate reader

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Academic Neurology Unit, Medical School, University Of Sheffield, Beech Hill Road, Sheffield, UK

- **Mitochondrial membrane potential measured in fibroblast cells**
- **Tetramethylrhodamine methylester fluorescence directly proportional to the membrane potential**
- **Performed on BMG LABTECH's FLUOstar® Omega in 96 and 384 well plate format**

Introduction

Measurement of the mitochondrial membrane potential is useful in a wide variety of research areas and mitochondrial dysfunction is implicated in diseases such as cancer, diabetes, Parkinson's disease, and stroke.
In most eukaryotic cells the majority of ATP production is via oxidative phosphorylation by the respiratory chain. In this way, sugars, such as glucose, and free fatty acids are oxidised, resulting in the pumping of protons across the inner mitochondrial membrane, creating an electro-chemical gradient (the mitochondrial membrane potential). This is in turn used by complex V of the respiratory chain to generate ATP. Therefore the mitochondrial membrane potential makes up a large part of the bioenergetic state of the cell and it is changed directly depending on the cells energy needs. For example fast growing tumour cells have a much higher mitochondrial membrane potential than WT cells, and in turn quiescent or differentiated cells have a still lower membrane potential.
The ability to accurately measure mitochondrial membrane potential can give invaluable information about the general health and function of the mitochondria, in particular of the overall function of the respiratory chain and the potential of the mitochondria to generate ATP and provide energy for other cellular components.
The number of factors which can influence this potential are vast and include many cellular components outside the mitochondria, for example interactions with the activity of the proteasome. In addition, the ability of a cell to maintain its mitochondrial membrane potential can mean the difference between survival of the cell, entry into an apoptotic cell death or a necrotic cell death process.Intact mitochondrial membrane potential is also required for the cell to enter apoptosis.
Mitochondrial membrane potential is usually measured using non-invasive cationic dyes. These dyes are sequestered into the mitochondrial matrix in amounts directly proportional to the membrane potential and can then be measured using standard fluorescent techniques, including fluorescence microscopy, flow cytometry or using a microplate reader for high through put assays.
BMG LABTECH's FLUOstar Omega microplate reader was used in a high throughput screening assay measuring mitochondrial membrane potential in human cells. The results presented here were gained using primary human fibroblast cells from controls.

Materials & Methods

- Human fibroblast cells obtained from Coriell Cell Repositories
- Black 96 or 384 well plates with transparent bottom from Greiner
- Tetramethylrhodamine methyl ester (TMRM), Sigma
- Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), Sigma
- Ethidium homodimer fluorescent dye, Invitrogen
- FLUOstar Omega microplate reader, BMG LABTECH

Fibroblasts were plated at 40 % confluency in 96 or 384 well plates in minimal growth medium MEM with 10 % FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM amino acids, 50 µg/mL uridine and 1 X MEM vitamins.
24 hours later cells were changed into galactose culture medium MEM (without glucose), supplemented with 10 % FBS, 100 UI/mL penicillin, 100 µg/mL streptomycin and 0.9 mg/mL galactose, as described before.
After further 24 hours of growth the mitochondrial membrane potential was measured using the fluorescent dye Tetramethylrhodamine methyl ester (TMRM) as described before. Briefly the dye was loaded onto cells at 150 nM in assay buffer (80 mM NaCl, 75 mM KCl, 25 mM D-glucose, 25 mM HEPES, pH 7.4), placed at 37°C for 5 minutes, washed 4 times in PBS and measured on a FLUOstar Omega microplate reader (Excitation: 544 nm and Emission: 590 nm, bottom reading with 50 flashes per well).
Initially TMRM is loaded into the cell via the plasma membrane and subsequently the TMRM dye is sequestered into the mitochondria as the mitochondrial matrix is the most negatively charged part of the cell. Therefore changes in plasma membrane potential could influence the results obtained using TMRM. In order to control for this, each assay is performed in parallel as above plus 10 µM carbonyl cyanide 3-chlorophenyl-hydrazone (CCCP), which collapses the mitochondrial membrane potential. All data is expressed as the total TMRM fluorescence minus the CCCP treated TMRM fluorescence.

It is important to quantify and control for cell number, especially when comparing results between cell lines, therefore cell number is measured using 1 µM ethidium homodimer fluorescent dye in a parallel plate after freeze thawing (Excitation: 544 nm and Emission: 645 nm, bottom reading with 50 flashes per well).

Rev. 07/2008
Keywords: **Bottom reading, CCCP, Ethidium homodimer, Fibroblasts, Mitochondrial membrane potential, TMRM**

Results & Discussion

Figure 1 shows the mitochondrial membrane potential in 6 control fibroblast cell lines, each measured in triplicate on separate occasions and showing the lack of variation between control cell lines.

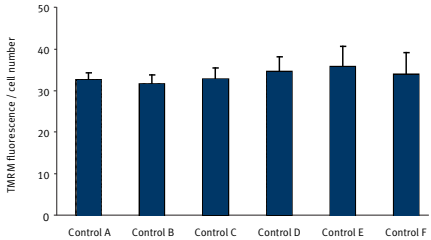

Fig. 1: Mitochondrial Membrane Potential measured using TMRM fluorescence in 6 different control fibroblast lines. The fluorescence values were corrected for the cell number.

Figure 2 shows the reduction in membrane potential of the same control fibroblasts after treatment with mitochondrial toxin 'X' for 72 hours. This demonstrates the range at which the assay is sensitive, creating a large 'therapeutic window'. Also in this figure the control cells have been treated with an experimental compound 'Y' which rescues the reduction in mitochondrial membrane potential caused by treatment with the mitochondrial toxin 'X'.

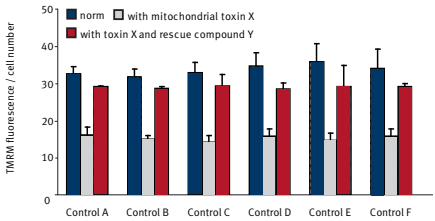

Fig. 2: Comparison of mitochondrial membrane potential in 6 untreated control fibroblast lines (norm), treated with mitochondrial toxin X alone and treated with mitochondrial toxin X and rescues compound Y. The fluorescence values obtained measuring TMRM were corrected for the cell number.

Figure 3 shows that the assay can reliably be scaled up to a 384 well plate format and gain robust, reproducible results.

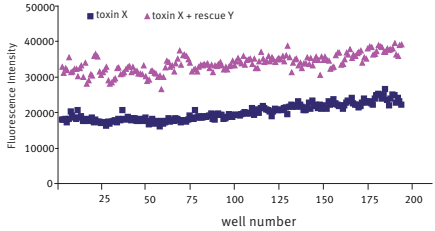

Fig. 3: Fluorescence intensity values indicating the membrane potential in control fibroblast in 384 well plate (192 wells used). The values were obtained either after treatment with mitochontrial toxin X alone or mitochondrial toxin X and rescue compound Y.

Conclusion

These results show that the FLUOstar Omega is suitable for measuring mitochondrial membrane potential in both 96 and 384 well plate formats. In addition, prolonged low dose treatment of healthy control fibroblast with a mitochondrial toxin causes a significant reduction in mitochondrial membrane potential.

The results are reproducible across a number of different control fibroblast lines and the results of the rescue of this defect with the experimental compound provide further evidence that this assay can be used for high throughput screening using BMG LABTECH's FLUOstar Omega.

PHERAstar® *FSX*
PHERAstar® *FS*

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Omega Series

Rapid ultra-sensitive isothermal DNA detection using RPA technology and a BMG LABTECH microplate reader

Olaf Piepenburg and Niall A. Armes
TwistDX Ltd., Meditrina Building, Babraham Research Campus, CB22 3AT Cambridge, UK

- **Isothermal nucleic acid amplification technology RPA is introduced**
- **Simple sample preparation procedure, constant temperature operation and homogenous format**
- **A BMG LABTECH microplate reader used for assay development**

Introduction

Nucleic acid amplification techniques (NAATs) are central to molecular DNA tests, including clinical diagnostics, environmental testing, food testing, and many other applications. While PCR-based testing in laboratories is well established, thermo-cycling equipment that can monitor reaction kinetics is expensive, as well as power-demanding and generally unsuitable in providing cost-effective access to a large number of end-users.

Expansion of the user market requires reduction in the cost complexity and power requirement of devices and a simplification of operating procedures. The isothermal nucleic acid amplification technology Recombinase-Polymerase-Amplification (RPA), recently developed by TwistDx, is ideally positioned to fulfil these requirements. The constant low reaction temperature of RPA, its resistance to temperature fluctuation, and the integration of the DNA amplification step with proprietary detection probes means that it can be employed easily using standard laboratory equipment such as a BMG LABTECH microplate reader as well as small portable devices anticipated in the near future.

Assay Principle

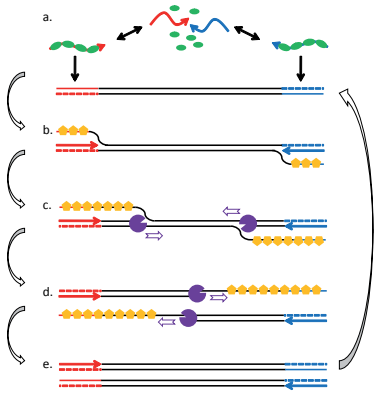

Fig. 1: RPA employs enzymes, known as recombinases (green), that are capable of interacting with amplification primers (red and blue arrows) and pairing them with homologous sequence in duplex DNA (indicated sections of black double line; a). Strand exchange and 'D-loop' formation is assisted by binding of single-strand DNA-binding protein (yellow) to the displaced strand (b). The 3' ends of the oligonucleotides are extended by strand displacing polymerases (purple), thereby copying the displaced strand (c, d). Both, the original template and the copy are then targets for subsequent recombination/extension events and an exponential amplification reaction is initiated (e). Using two gene-specific primers, the recombinase-driven RPA process ensures that DNA synthesis is directed only to defined target sites in a given template molecule.

The technology achieves amplification from single template molecules to detectable levels in very short time-frames (down to less than 10 min) at a constant and low temperature.

Crucially for its application in non-laboratory settings, RPA can be combined with very simple sample preparation procedures and can utilise stabilised reaction components. Moreover, by employing a novel and proprietary fluorescent probe system, RPA can operate in a homogenous detection format that facilitates both, ease of application and great read-out speed.

Probes for the RPA system use a fluorophore and quencher separated by a nuclease target site which operates only on duplex DNA – thus fluorescence is seen to increase when specific amplification has occurred.

In this application note we describe the use of a BMG LABTECH microplate reader for fluorescence intensity measurements as a means of real-time monitoring of DNA detection reactions using RPA.

Materials & Methods

Reagents

The proteins used in this experiment were purified as described previously, except Creatine Kinase, which was obtained from Roche. PEG35000 was obtained from Calbiochem. Oligonucleotide amplification primers and probe (containing an internal FAM fluorophore and a corresponding BHQ1 quencher) were supplied by Eurogentec.

Lyophilisation

Complete RPA reaction components including probes and primers were lyophilised in microfuge tubes in a Virtis Genesis freeze dryer.

Sample preparation

Template material was prepared from buccal swaps by incubation in 0.3 M NaOH for 5 min at room temperature. The obtained lysis solution was used directly in amplification reactions. Control template (female and male human genomic DNA) was obtained from Promega.

Reaction set-up

For each reaction a rehydration solution containing buffer components and the template was added to lyophilised reagents and mixed, bringing the total volume of each reaction to 50 µL. The template portion of the solution consisted of water or 300 copies human genomic DNA for the controls (female or male DNA; note that this corresponds to 150 copies of the Y-chromosome) or 1.5 µL sample material. The completed reactions were transferred to a black 384-well plate with clear bottom from Greiner and read at 37°C.

Rev. 08/2008
Keywords: **Amplification, DNA quantification, Nucleic acids, PCR, Recombinases**

Results & Discussion

The target for this validation experiment is located within the sex-determining region on the human Y-chromosome (Sry), and the amplification primers and detection probe were designed accordingly.

A total of 9 samples were tested for the presence of the Sry target sequence on the human Y chromosome. These included a 'no template' control (water), a positive control (male human genomic DNA), a negative control (female human genomic DNA), and material from buccal swaps obtained from 6 individuals (5 male, 1 female).

Reactions were set up as described under Material and Methods and monitored at 37°C for a total reaction time of 20 min. Readings were taken every 30 sec, following a 2 sec shaking cycle (double-orbital, 1 mm), and using excitation and emission filters of 485-12 nm and 520-10 nm wavelength, respectively. As shown in Figure 2 both negative controls and the sample prepared from a female buccal swap did not result in the generation of a fluorescent signal.

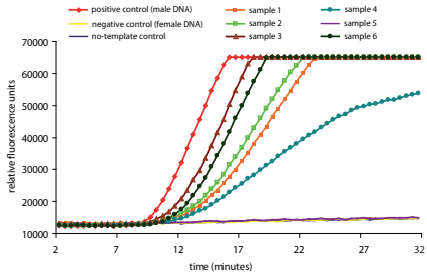

Fig. 2: A total of 3 controls and 6 samples were tested for the presence of the Y-chromosome target. The two negative controls and the sample derived from the buccal swap of a female individual (sample 5) did not produce a fluorescent signal above the baseline. The positive control and all swaps from 5 male probands generated a positive signal within 12 minutes of the start of the reaction. Note that the onset of amplification in positive control (300 copies of template DNA in the reaction, corresponding to 150 copies of the Y-chromosome) precedes the 5 test samples, indicating that the total amount of template in samples 1 to 5 is probably lower than 300 genomic copies.

By contrast, the positive control and all 5 male buccal swaps scored positive for the presence of the Sry target within less than 12 min of initiation of the reaction. The exact number of template molecules in the different sample preparations is likely to vary as buccal samples were not quantified, probably accounting for the differences in the time of onset of amplification and in the total signal strength between the six sample reactions.

Conclusion

The experiment described in this application note shows the detection of a target sequence on the Y-chromosome in sample material prepared from buccal swaps, thus validating the feasibility of combining all important aspects of a working NAAT in a single integrated format: a very simple sample preparation procedure, a DNA amplification system requiring isothermal temperature conditions and a homogenous read-out system.

Furthermore, the established assay delivers the test reagents in a stabilised and easy–to-use formulation, demanding only very limited user input for operation (two pipetting/mixing steps), lending itself for deployment in non-laboratory settings. Eventually, the implementation of the technology in point-of-care and field-testing environments will require the use of compatible and cost-effective handheld fluorescent reading devices. However, the BMG LABTECH microplate reader acts as a valuable tool for both assay development programs and for the use of the TwistDX technology platform in laboratory and high-throughput applications.

Background

TwistDX is a biotechnology company located on the Babraham research campus near Cambridge, UK. The focus of the company lies in the integration of its proprietary nucleic acid amplification and detection technologies with user-friendly sample preparation procedures and test delivery formats, enabling portable DNA testing assays for the point-of-use market. TwistDx will also provide DNA amplification and detection alternatives for laboratory based nucleic acid testing in both, the research & development and applied markets in the near future.

Fluorometric determination of extracellular enzyme activities in peat using a BMG LABTECH microplate reader

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Institute for Sustainable Water, Integrated Management and Ecosystem Research, University of Liverpool, Merseyside, UK

- **Fluorescence method to measure extracellular enzyme activities in peat**
- **Methylumbelliferone (MUF) and derivatives used as substrates**
- **Fast measurement in 96-well format using a BMG LABTECH microplate reader**

Introduction

Peatland ecosystems represent a significant global store of carbon (C) due to the historic accumulation of organic matter resulting from suppressed microbial de-composition. Decomposition is limited by extracellular enzymes that are produced by the plant and microbial communities and function independently of the microbial community. The storage of carbon in peatland ecosystems is therefore regulated in part by the environmental factors that determine extracellular enzyme activities. Understanding the environmental controls and processes that regulate extracellular enzyme activities is therefore critical in determining the fate of carbon in peatland ecosystems.
Here we present the results from a method developed to measure potential extracellular enzyme activities in peat using a BMG LABTECH microplate reader. The assay is based on the use of fluorogenic methylumbelliferyl (MUF) substrates that fluoresce upon enzymic cleavage allowing the amount of product to be measured.

Materials & Methods

- Black 96-well microplates, F-bottom, Greiner Bio-One
- MUF and MUF substrates, Sigma-Aldrich
- BMG LABTECH microplate reader

Peat samples were collected from Langdon moor, Durham, UK in order to determine the concentration of methylumbelliferyl (MUF) substrates to be added to peat solutions to achieve enzyme 'active-site' saturation and the optimal assay incubation length.
The hydrolytic enzymes β-glucosidase, cellobiohydrolase, and N-acetylglucosaminidase (or chitinase) were determined using MUF artificial substrates 3 (see Table 1). For each peat replicate sample, ten cm3 of peat was placed in a 50 mL centrifuge vial and deionised water added up to the 50 mL mark. The vial was shaken by hand for 30 seconds and mixed using a vortex for a further 30 seconds. Using a sawn-off pipette tip, 0.75 mL of peat slurry was placed into a 1.7 mL centrifuge vial. To the vial, 0.75 mL of MUF substrate was added and samples were incubated at field temperature for one hour. Samples were centrifuged for 5 minutes at 12,000 rpm and 300 µL of supernatant transferred to a well in a plate. Fluorescence was determined on a BMG LABTECH microplate reader at 330 nm excitation and 450 nm emission wavelength. Enzyme activities were determined from the fluorescence units using a standard calibration curve of methylumbelliferone (MUF) and expressed as rates of MUF production (µmol MUF per g^{-1} dry peat weight per min^{-1}). Fluorescence quenching is a potentially interfering process which decreases the intensity of the fluorescence emission and occurs in water containing peat-derived compounds. The standard calibration curve accounted for quenching by dissolving the MUF standard in 150 µL of centrifuged peat slurry for each peat replicate.

Table 1: Enzymes and enzyme MUF substrates.

Enzyme	MUF substrate	Reaction
Cellobiohydrolase	MUF-β-D-cellobioside	Cellulose polymers to dimers
β-glucosidase	MUF-β-Dglucopyranoside	Cellulose dimers to monomers
Chitinase	MUF-N-acetyl-β-Dglucosaminide	Chitin to acetylglucosamine

The assay above was performed with substrate concentrations varying from 0 to 500 µM in order to determine the optimal substrate concentration. Using the optimal substrate concentrations, samples were then incubated for varying lengths of time to determine the optimal assay incubation length.

Results & Discussion

Figure 1 shows the effect of increasing substrate concentration on the activity of cellobiohydrolase. Activity followed Michaelis-Menten kinetics and reached saturation at approximately 200 µM of substrate.

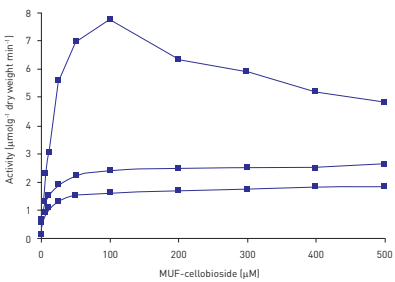

Fig. 1: Effect of substrate concentration on cellobiohydrolase activity (n = 3).

One of the replicates exhibited substrate inhibition at 100 µM of substrate resulting in a relative standard deviation of 85 % at 100 µM of substrate. This is mainly due to spatial variation of enzyme activity within the block of peat being analysed, NOT analytical variation.
Figure 2 shows the effect of increasing substrate concentration on the activity of β-glucosidase. Activity followed Michaelis-Menten kinetics and reached saturation at approximately 200 µM of substrate. One of the replicates exhibited substrate inhibition at 100 µM of substrate resulting in a relative standard deviation of 91 % at 100 µM of substrate.

Rev. 09/2008
Keywords: **Beta-glucosidase, Cellobiohydrolase, Chitinase, Enzyme activity, Peat**

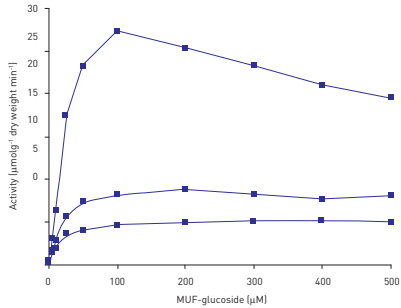

Fig. 2: Effect of substrate concentration on β-glucosidase activity (n = 3).

Figure 3 shows the effect of increasing substrate concentration on the activity of chitinase. Activity followed Michealis-Menten kinetics and reached saturation at approximately 300 µM of substrate.

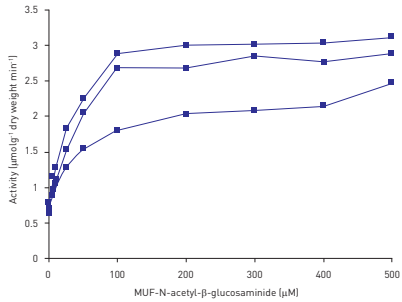

Fig. 3: Effect of substrate concentration on chitinase activity (n = 3).

Figure 4 shows the significant quenching effect of peat on fluorescence.

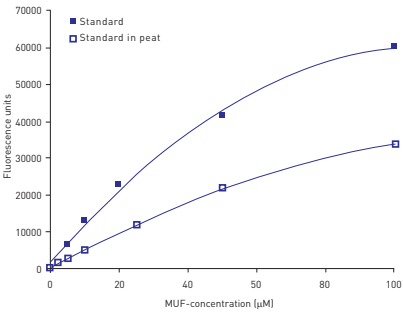

Fig. 4: The quenching effect of peat on fluorescence.

Time course incubations performed using optimal substrate concentrations for cellobiohydrolase, β-glucosidase and chitinase showed that one hour was the maximum amount of time that reaction rates remained linear (data not shown).

Conclusion

The extracellular enzymes β-glucosidase, cellobio-hydrolase and N-acetylglucosaminidase reached substrate saturation at 200 µM, 200 µM and 300 µM of MUF respectively. Measured enzyme activities were linear for up to 120 minutes although 60 minutes duration of incubation was chosen for future applications to minimise variation between replicates. One replicate exhibited substrate inhibition for the β-glucosidase and cellobio-hydrolase assays.

The results show that potential extracellular enzyme activities can be determined in peat at low cost and within a short period of time using a BMG LABTECH microplate reader.

Application of the Amplifluor® SNPs genotyping system using a BMG LABTECH microplate reader

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- **SNPs were easily detected with the Amplifluor® System from Millipore**
- **Detailed protocol and instrument settings are shown for this assay**
- **The BMG LABTECH microplate reader has proved to be an excellent reader for this assay**

Introduction

SNPs (Single nucleotide polymorphisms) are positions in genomic DNA with a single nucleotide difference between individuals of a species. SNPs are the most frequent mutations found in DNA sequences. These small genetic variations can occur in coding and non-coding regions of the genome and can either have an extreme or no effect on a particular organism. Therefore SNPs and their detection are important for medical research and pharmacology.
The detection of SNPs (SNP Genotyping) can be achieved with a number of different methods. In this application note we describe a homogeneous fluorescence assay based on PCR. The Amplifluor® SNPs HT Genotyping System from Millipore was used to screen a number of samples with the help of a BMG LABTECH microplate reader.

Assay Principle

The method utilises two fluorescently labeled oligonucleotides that consist of a specific 3' primer sequence region and a 5' hairpin region that is labeled with either of two fluorophores (FAM or JOE).
The presence of the 3' primer sequence enables these fluorescent oligo-nucleotides to work with target specific unlabeled primers designed to the researcher's specific target sequence. Thus, the method can be used for any variation of bases. (Fig.1)

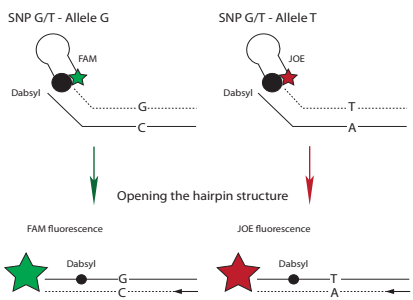

Fig. 1: Principle of the Amplifluor® SNPs Genotyping System after addition of FAM and JOE coupled primers. Scheme is based on Millipores' patented method.

The researcher simply designs the allele specific primers containing 5' tails that match the 3' tails on the fluorescently labeled primers. In the example depicted in Fig. 1, the FAM labeled primer is G-specific whereas the JOE labeled primer is T-specific. A quencher dye (Dabsyl)is held within close proximity of each fluorophore by the 5' hairpin region of the oligonucleotide. After successful incorporation of the primers, the hairpin region is opened and the Dabsyl loses its ability to quench the fluorophores, resulting in a large fluorescence signal. The fluorescence produced during each PCR cycle directly correlates to the amount of amplified DNA.

Materials & Methods

- Amplifluor® SNPs Genotyping System, Millipore
- Platinum® Taq DNA Polymerase, Invitrogen
- ABgene 1100 Thermo-Fast® PCR plate, Thermo Scientific
- BMG LABTECH microplate reader

All reagents needed for the amplifcation reaction (except polymerase) should be thawed from -20°C storage. DNA (5-150 ng/µL) that has been diluted in distilled water or TRIS-buffer (but not TRIS-EDTA buffer) is placed in a separate rack. All reagents are mixed thoroughly and vortexed briefly before preparing the amplification mixture. The cocktail of reagents is prepared at room temperature in a sterile 1.5 mL microcentrifuge tube. All reagents are added in the order presented in table 1. The reagents should be mixed with a pipette prior to dispensing.

Table 1: Amplification Cocktail Preparation: Reaction Volume -10 µL.

Reagent	1 Rxn [µL]
dH2O	3.15-4.15
dNTPs (2.5 mM each)	0.8
20x amplifluor SNP FAM Primer	0.5
20x amplifluor SNP JOE Primer	0.5
20x SNP specific Primer Mix	1-2
10x Reaction Mix S Plus	1.0
Platinum Taq (5U/µL) add at last minute	0.05
TOTAL	**8.0**

DNA samples (2 µL for 10 µL reaction mix) are then transferred into a PCR plate. For mutant amplifluors; mutant, wild type, heterozygote and no DNA controls are used. The polymerase is then added to the amplification cocktail, mixed and spun down. 8 µL of the amplification cocktail is added to each DNA sample/control. The plate is sealed with optically clear strips and then placed into a thermocycler and the PCR program is started.
For some assays a total reaction volume of only 5 µL will also work. The volumes of individual reagents however, need to be adjusted accordingly.

Rev. 03/2010
Keywords: **Amplifluor, Bottom reading, Genotyping, PCR, Polymorphism, SNP genotyping**

PCR Program

1. 95°C 4 min
2. 95°C 10 sec
3. 60°C 20 sec
4. 72°C 40 sec
5. Go to step 2 for 34 times cycles
6. 72°C 3 min
7. 10°C hold

After completion of the PCR procedure the plate is cooled to room temperature. The plate can either be read stored at 4°C or spun down and placed into the BMG LABTECH microplate reader for reading. The instrument is set to read FAM (Excitation 490-10 and Emission 520-10 nm) and JOE (Excitation 530-10 and Emission 560-10 nm) in bottom reading mode.

BMG LABTECH reader settings

Measurement Mode: Fluorescence intensity
Reading Mode: Endpoint / flymode
No. of flashes per well: 3
Optic Settings: multichromatic; bottom reading

No.	Excitation	Emission
1	490-10	520-10
2	530-10	560-10

NB: For Omega readers, you need to use 3 spacers for ABgene 1100 PCR plates.

Results & Discussion

After measurement the data is transferred into an Excel Macro that can be downloaded at the Millipore homepage. A typical result is shown in figure 2.

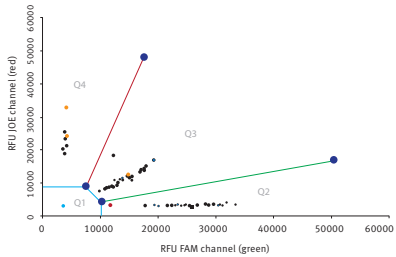

Fig. 2: A typical pattern of samples. The blue dot in Q1 is buffer. The red dot in Q2 shows the mutant control(green allele), Yellow dots in Q4 are wild type control(red allele) and the orange dot in Q3 represents the heterozygote control(red green allele). Figure 2 clearly shows that all controls that are homozygous for either allele or heterozygous or a mixture only are clearly separated. In addition, samples are precisely located in their clusters.

Conclusion

High S/B values indicate that the BMG LABTECH microplate reader is an excellent instrument for running this assay. It is easily capable of measuring the output from the Amplifluor SNP Genotyping assay kit (Millipore) with high precision and with high throughput.

A leukocyte adhesion assay performed on a BMG LABTECH microplate reader

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- **Leukocyte adhesion assay utilized to screen for potential inflammatory markers**
- **BMG LABTECH microplate reader used for cell based bottom optic measurements and well scanning**
- **Results show good reproducibility and can be adapted to e.g. primary neutrophils**

Introduction

A key component of the inflammatory response is the recruitment and extravasation of leukocytes into perivascular tissue. This process requires leukocyte interaction with vascular endothelium and consists of a multistep process including the capture of circulating leukocytes, subsequent leukocyte rolling, arrest, firm adhesion and transmigration (Figure 1).

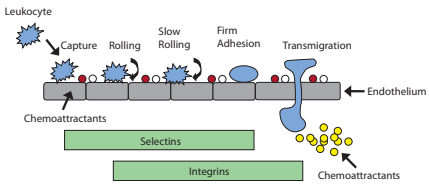

Fig. 1: Processes involved in leukocyte capture, adhesion and transmigration across the endothelium.

This complex process initially involves the recognition and interaction of endothelial cell adhesion molecules with their specific ligands on leukocytes. The capture and the removal of the leukocytes from the flowing blood, as well as the subsequent rolling of the leukocyte along the vessel wall, are due to the reversible binding of selectins which are found on both the leukocytes and the endothelial cell surface. Arrest and firm adhesion of leukocytes to endothelium is dependent on the activation of β2 integrins including Mac-1 or LFA-1 on the leukocyte cell surface, followed by interaction with endothelial cell proteins belonging to the immunoglobulin superfamily such as ICAM-1.

We have used gene regulatory networks to identify novel regulatory hubs genes involved in the inflammatory response in human umbilical endothelial cells (HUVECs). To assess the potential role of these genes we have used a leukocyte adhesion assay to model this complex process and looked at the response of the endothelium following RNAi knockdown of the selected genes. We have used primary peripheral blood mononuclear cells (PBMCs) and a number of leukocyte cell lines (HL60, JY and U937) which differ in their adhesion ligands.
All measurements were done using a multimode microplate reader from BMG LABTECH.

Materials & Methods

The materials were purchased from the following manufacturers:

- BMG LABTECH microplate reader
- siRNA on-target reagents from Dharmacon, Perbio
- Cell tracker Green dye from Invitrogen
- Microplates, black 96-well from Greiner
- Tissue culture media from Lonza
- HL60.Ast and JY cell lines from ECACC
- TNFα recombinant protein from R&D systems

HUVECs were grown in EGM2 culture media and were combined into pools of cells from 10 donors for each experiment. Cells were grown for 24hrs following recovery from frozen storage and plated into T25 tissue culture flasks. Transfections were carried out using 100 nM of siRNA on-target plus pools, 6 hrs after transfection the cells were re-seeded into 96-well plates. To allow knockdown of the target protein the cells were left for 48 hrs post transfection in normal growth conditions. The plates were then gently washed using a multichannel pipette. EGM2 media +/-TNFα was added to selected wells. The plates were incubated for 4 hrs to allow upregulation of cell adhesion molecules, and then washed gently once with PBS.

Whilst incubating the HUVECs with TNFα, the HL60 cells (or other leukocyte cells) were labelled with cell tracker green (2.5 µM in PBS) for 25 min at 37°C following the manufactures instructions. After labelling and washing the HL60 were resuspended in EGM2 and then added to all wells ($1x10^5$/well) except the negative control wells to which unlabelled cells were added.

The plates were incubated for 1 hr to allow leukocyte adhesion and then washed gently x3 with PBS at RT to remove any unbound cells. Fluorescence in each well was then quantified using the BMG LABTECH microplate reader (Excitation 485 nm and emission 520 nm) with replicates of 5 per sample. Knockdown of the TNFRSF1A was used as a positive control.

Results & Discussion

Initial results demonstrated that there was a linear relationship between cell number and fluorescence (Figure 2).

Rev. 03/2009
Keywords: **Adhesion, Cell tracker green, Leukocytes, TNFα, Inflammatory response, Well scanning**

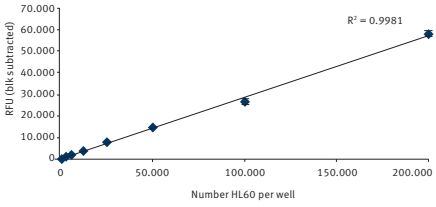

Fig. 2: Cell tracker green labelling of HL60 cell line showing linear relationship to cell number in 96 well plates.

Preliminary adhesion assays were then set up using HUVECs that were transfected with siRNA for luciferase (siControl) and TNFRSF1A (positive control). 48 hrs after transfection the cells were treated with TNFα (0-10 ng/mL) for 4 hrs prior to the addition of cell tracker green labelled HL60 cells. Following 1 hr incubation and gently washing to remove unattached HL60, the fluorescence was quantified using the BMG LABTECH microplate reader in fluorescence mode with bottom optic measurement. Results shown in Figure 3 demonstrate that increasing the concentration of TNFα causes increased adhesion of HL60 to the HUVEC monolayer in both untreated and siControl KD conditions. However following siRNA knockdown of the TNFRSF1A there is no significant effect of TNFα on HL60 adhesion. This would be predicted as this receptor is required for TNFα signalling.

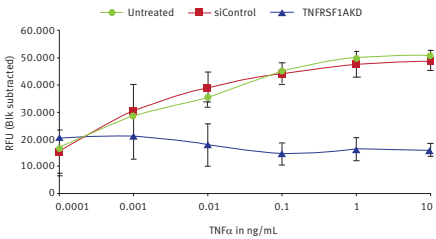

Fig. 3: Adhesion of Cell tracker green labelled HL60 cell line to TNFα treated HUVEC following siRNA gene knockdown (TNFRSF1AKD).

QPCR studies confirm that the knockdown efficiency was >90%. This is a very valuable positive control for this assay and was used for all subsequent studies. The well scanning mode of the BMG LABTECH microplate reader is helpful when scaling this assay up to use in 48 well plates as it ensures accurate quantification due to the possible uneven distribution of the HL60 cells. This function also provides a good visual output for the assay in a 96-well plate as shown in Figure 4 below.

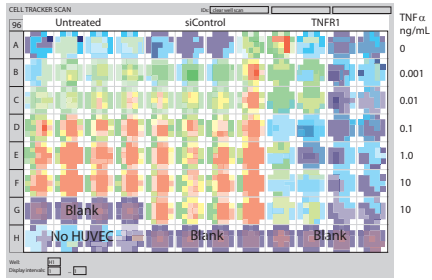

Fig. 4: Well scanning view of cell tracker green labelled HL60 adhesion to TNFα stimulated HUVEC. (Blank wells contain unlabelled HUVECs in media, the "no HUVEC" wells contain labelled HL60 to check adhesion to well surface).

The results were compared with fluorescent microscopy analysis of the wells (data not shown). This showed a very similar pattern but use of the microplate reader enables rapid analysis of multiple plates rather than the time consuming image capture and quantification by microscopy.

Conclusion

The results show good reproducibility for a complex biological assay and provide a method for rapid screening of this aspect of the inflammatory process. We have utilized this method to screen a number of potential inflammatory markers and it provides a very easy to use technique which can also be adapted for primary neutrophils or PBMC if a more "in vivo-like" assay is required.

FRET-based screening for potential modulators of the Gαi1 protein/GoLoco interaction

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- **CFP-YFP FRET based assay utilized to screen for potential inhibitors of $G\alpha_{i1}$/GoLoco interaction**
- **Inhibitors of the G protein/GoLoco protein interaction are putative pharmacological tools**
- **The POLARstar® Omega allows for fast screening and easy data evaluation**

Introduction

Many extracellular signals, including hormones, neurotransmitters, and growth factors, are relayed intracellularly by binding the extracellular portion of a seven-transmembrane domain (7TM) receptor that is coupled to an intracellular heterotrimeric G protein. The stimulated G-protein coupled receptor (GPCR) propagates the extracellular signal through activation of the alpha subunit of heterotrimeric G proteins (Gα), which occurs when Gα exchanges GDP for GTP. The Gα subunit is also modulated by a family of proteins containing the GoLoco motif, which serves to inhibit the exchange of GDP for GTP by the Gα subunit. The G protein/GoLoco interaction is therefore an attractive pharmacological target because it serves as a point of regulation for downstream GPCR signaling.

Presently, no small molecule inhibitors of the Gα/GoLoco interaction are available for study. Although there is currently a lack of inhibitors for this recently discovered class of proteins, the potential applications of a GoLoco inhibitor are significant. Combining existing GPCR agonists with specific GoLoco motif inhibitors could synergistically increase the specificity of existing drugs and serve as useful chemical probes.
To identify and assess potential modulators of the Gα/GoLoco interaction, putative inhibitors were screened in a FRET (Fluorescence/Förster Resonance Energy Transfer) assay using BMG LABTECH's POLARstar Omega.

Assay Principle

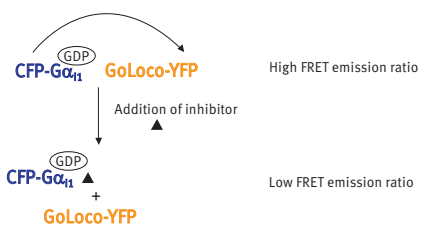

Fig. 1: Assay Principle for the FRET-based inhibitor screening.

The ground state Gai1 binds GDP. The GoLoco motif selectively recognizes $G\alpha_{i1}$ (GDP) and binds with nanomolar affinity. In a FRET-based assay which measures the Gα/GoLoco interaction, the FRET partners CFP-$G\alpha_{i1}$·GDP and YFP-GoLoco yield a significantly increased (530/485 nm) FRET ratio when bound to each other than when compared with the non-interacting state of CFP-$G\alpha_{i1}$ (as formed using aluminum tetrafluoride which mimics the transition state for GTP hydrolysis).
An inhibitor of this interaction could possibly perturb or displace the nucleotide from the Ga binding pocket or it could occlude the GoLoco motif binding surface, thus preventing the protein/protein interaction. When Gα and GoLoco are no longer interacting, the (530/485 nm) FRET ratio decreases (Figure 1).

Materials & Methods

- Black polystyrene 96-well plates, Corning
- YFP: amino acids 1-237 of pEYFP-C1 open reading frame (ORF), BD Biosciences Clontech
- CFP: amino acids 1-239 of pECFP-C1 ORF, BD Biosciences Clontech
- POLARstar Omega, BMG LABTECH

The RGS12 Ga·GDP binding region (GoLoco motif, aa 1187-1221) was produced as a fluorescent chimera with the yellow fluorescent protein (YFP). Compounds were titrated into an optimized concentration of YFP-RGS12GoLoco and cyan fluorescent protein labeled $G\alpha_{i1}$(CFP $G\alpha_{i1}$) to confirm inhibitor activity. Ground state $G\alpha_{i1}$ is GDP bound and the GoLoco motif binds readily, thus producing a higher emission ratio in the FRET assay from the non-directly excited fluorophore. In the mimicked GTP hydrolysis transition state, created by the binding of aluminum tetrafluoride (denoted AMF), the $G\alpha_{i1}$/GoLoco interaction does not occur. This is evident by the decrease in the observed FRET ratio of non-directly excited fluorophore emission versus directly excited fluorophore emission.
For FRET measurements, the POLARstar Omega was used with filters optimized for CFP/YFP FRET. Excitation filter with wavelength at 422 nm and dual emission wavelength filters at 530 nm and 485 nm were used. Instrument settings can be found below.

Instrument settings

Positioning Delay	0.2 sec
Measurement start time	0.0 sec
No. of flashes per well	10
No. of multichromatics	1
Simultaneous dual emission activated (only sequential measurements available on CLARIOstar and FLUOstar Omega)	
Excitation filter	422 nm
Emission A filter	530 nm
Emission B filter	485 nm

The FRET pair (100 nM CFP-$G\alpha_{i1}$ and 100 nM YFP-RGS12GoLoco) in either buffer (GDP or AMF buffer) was

Rev. 09/2009
Keywords: **CFP-YFP, GPCR, Inhibitor screening, Receptor, Simultaneous Dual Emission SDE, Transmembrane**

aliquotted in duplicate 60 µL volumes with increasing concentrations of non-labeled competitor. The plate was read immediately and the FRET ratio (530/485) determined using the MARS Data Analysis Software.

GDP Buffer: 10 mM Hepes pH 7.4, 150 mM NaCl, 0.0005 % NP40 alternative, 100 µM GDP, 50 µM EDTA
AMF Buffer: 10 mM Hepes pH 7.4, 150 mM NaCl, 0.0005 % NP40 alternative, 100 µM GDP, 50 µM EDTA, 10 mM $MgCl_2$, 10 mM NaF, 30 µM $AlCl_3$

Results & Discussion

The G-protein/GoLoco interaction is dependent on the nucleotide bound state of the G-protein. The interaction occurs with the GDP bound state of the G-protein, but not in the GTP hydrolysis-mimicking state (AMF). Figure 2 shows the FRET ratio obtained in both buffers for different concentrations of unlabeled competitor $G\alpha_{i1}$.

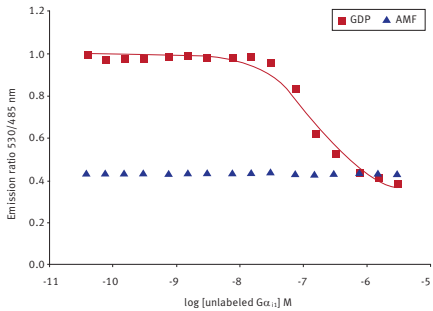

Fig. 2: 530/485 nm emission ratio of CFP-$G\alpha_{i1}$ and YFP-RGS12GoLoco measurements using different buffers (GDP-buffer and AMF buffer) and varied concentrations of unlabeled competitor G-protein.

A Z' value of 0.853 was obtained when the reaction volume was at 60 µL. The assay system also demonstrated a robust tolerance to DMSO (up to 5%), a commonly used solvent for small molecule screening (Figure 3).

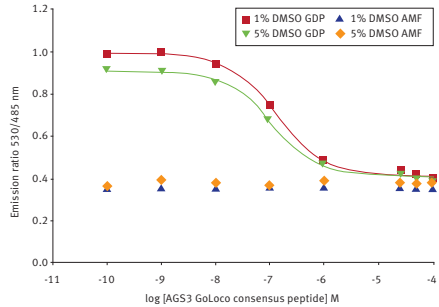

Fig. 3: DMSO tolerance of CFP-Gai1 and YFP-RGS12GoLoco measurements in GDP and AMF buffer with competitive inhibition of the interaction using varied concentrations of unlabeled GoLoco motif peptide.

The CFP/YFP FRET based assay is a robust assay and was used in a medium-throughput small molecule screen of 57 compounds (Figure 4).

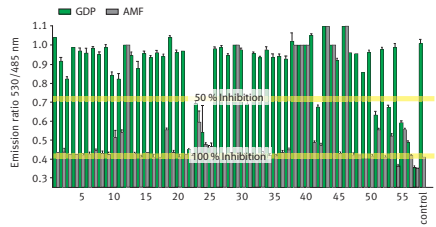

Fig. 4: GoLoco Screen of 57 compounds in GDP and AMF buffer.

Conclusion

The results show that the CFP-YFP FRET based assay is a robust assay that tolerates DMSO in concentrations often used to dissolve small molecules. A very good Z' value of 0.853 in a low volume of only 60 µL shows the potential to perform the assay in 384-well format.
The POLARstar Omega proved to be a reliable and robust instrument to perform these FRET-based measurements. With the MARS Data Analysis Software, the FRET ratio and Z' value are automatically calculated providing fast and efficient evaluation.

alamarBlue® assay for assessment of cell proliferation using a BMG LABTECH microplate reader

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- **alamarBlue® cell proliferation assay is based on a reducing environment that indicates metabolic active cells**
- **Cell proliferation on different substrates was followed over 17 days with a BMG LABTECH microplate reader**
- **Fluorescent and colorimetric detection possible**

Introduction

A variety of methods have been developed to measure cell proliferation in whole population of cells. These include the detection of antigens by immunohistochemistry, measurement of DNA synthesis, and quantification of the reducing environment of the cells. The latter relates to the fact that when cells are metabolising they maintain a reducing environment within their cytosol and this reduced state can be measured spectrophotometrically through the conversion of fluorometric/colorimetic REDOX indicators. This application note focuses on quantification of the reduction of the intracellular environment by alamarBlue®.
The reducing environment of the cells in the alamarBlue® assay is measured through the conversion of resazurin (oxidised form) to resorufin (reduced form) (Figure 1).

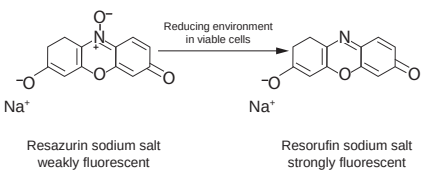

Fig. 1: alamarBlue® assay principle.

This results in colorimetric (absorbance) and fluorescence changes. Resazurin is blue and non-fluorescent whereas resorufin is red and highly fluorescent. It is a sensitive assay if working with higher than $5x10^3$ cells per 100 µL, relatively non-toxic and provided that it is carried out carefully, the same replicates can be followed over several time points. The disadvantage of this assay is that it is not a direct cell counting technique like heamocytometry, because the fluorescence or absorbance signal can be affected by both changes in cell number and cell metabolism.

Materials & Methods

- 96 well black polystyrene microplates (Greiner Bio-One)
- alamarBlue® (MorphoSys UK)
- BMG LABTECH microplate reader

Early passage Human Osteoblast cells (HOb 406-05a), supplied by the European Collection of Cell Cultures ECACC were seeded onto substrates A and B at a density of 10^4 per sample. The samples were each placed in a well of a 24-well plate, covered with 1 ml of culture medium and incubated for a total of 17 days. The culture medium was McCoy's 5A medium containing 10% heat inactivated serum, 1% glutamine and vitamin C (30 µg/mL).

To measure cell proliferation, the seeded substrates were incubated for 4 hours (longer incubation times may be used for greater sensitivity) with fresh culture medium supplemented with 10 vol% alamarBlue®. A total of 10 replicates were used for each substrate.

Following incubation, 100 µL medium from each well was transferred to a 96-well black polystyrene microplate and replicated 3 times. Fluorescence (excitation 530 nm, emission 590 nm) was measured on a BMG LABTECH microplate reader. (Note: Colorimetric detection can be monitored by absorbance measurements at 570 nm, while using 600 nm as a reference wavelength. However, it tends to be less sensitive because there is considerable overlap of the oxidized and reduced forms of the alamarBlue® when measured by absorbance).
The alamarBlue® reduction by the cells expressed as fluorescence emission intensity units was measured on days 1, 3, 6, 8, 10, 13, 15 and 17 of the culture period for different samples. The % reduction of alamarBlue® for each case was calculated using the formula

$$\text{percentage reduction of alamarBlue®} = \frac{S^x - S^{control}}{S^{100\%\ reduced} - S^{control}}$$

where SX is the alamarBlue® fluorescence signal of the sample at day x, $S^{100\%reduced}$ is the signal of the 100% reduced form of alamarBlue® and $S^{control}$ is the signal from the control: the culture medium supplemented with 10 vol.% alamarBlue®. The 100% reduced form of alamarBlue® was produced by autoclaving controls (ie. culture medium supplemented with 10 vol.% alamarBlue®) at 121°C for 15 minutes.

Results & Discussion

Fig. 2 shows the percentage reduction of alamarBlue® after being averaged and including the standard deviations. Greater alamarBlue® reduction (i.e. higher levels of cell growth) is observed for substrate A over the whole culture period. In both substrates, cell proliferation increased with culture time over the first 15 days. The metabolic activity of the cells growing on both substrates seems to slow-down by day 15, suggesting that the surfaces were advancing into confluence.

Rev. 06/2009
Keywords: **AlamarBlue, Cell proliferation, Resazurin, Resorufin, Viable cells**

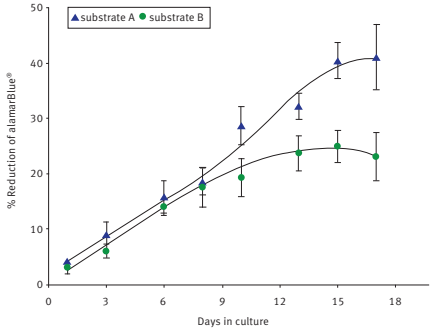

Fig. 2: Percentage reduction of alamarBlue® as a function of culture time, for substrates A and B.

Conclusion

This application note shows that the reducing environment of cells can be accurately monitored using a BMG LABTECH microplate reader. The attraction of the alamarBlue® assay is that it incorporates a nontoxic reagent which allows continuous monitoring of cell proliferation on the same samples using either fluorescence or absorbance.

BMG LABTECH's readers are flexible multifunctional microplate readers that have six different measurement modes in one instrument: fluorescence intensity, time-resolved fluorescence, luminescence, fluorescence polarization, AlphaScreen® and absorbance. With two optional onboard injectors and a standard 45°C incubation chamber, BMG LABTECH microplate readers can easily become fully automated to perform any cell-based assays.

Using intrinsic tryptophan fluorescence to measure heterotrimeric G-protein activation

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- **Heterotrimeric G protein activation is measured via the change in tryptophan fluorescence in the Gα subunit**
- **This method applies to most $G\alpha_i$, $G\alpha_o$ and some small G proteins that have movement in the switch II region**
- **Alternative, non-radiological method to ^{35}S-GTPγS and[γ-^{32}P]GTP assays**

Introduction

GTP-binding proteins (G-proteins) are important, well-described cellular signaling molecules. Heterotrimeric G-proteins are composed of three subunits Gα, Gβ and Gγ and are typically bound to seven trans-membrane G-protein coupled receptors (GPCRs). The Gα-subunit binds guanine nucleotides while the Gβ and Gγ subunits form an obligate heterodimer. In its inactive state, the GDP bound Gα subunit is bound to Gβγ. Upon agonist activation the receptor acts as a guanine nucleotide exchange factor (GEF), resulting in the release of GDP and subsequent binding of GTP. The binding of GTP causes a dramatic conformational change in three flexible switch regions of Gα (Fig. 1 dark red and dark blue) resulting in the dissociation of Gα-GTP from Gβγ.

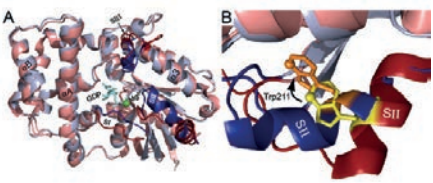

Fig. 1: A) Structural models of $G\alpha_{i1}$ (an isoform of Gα) bound to GDP (red) and GDP · AlF_4^- (blue) (PDB ID: 1KJY and 2IK8, respectively). The switch regions SI, SII, and SIII are in dark red and blue. (B) Close-up view of intrinsically-fluorescent Trp211 located in the switch II region in inactive (yellow) and activated (orange) $G\alpha_{i1}$.

The duration of activation is controlled by the hydrolysis rate of GTP. Two well-described accessory protein families affect the kinetics of Gα subunits by either accelerating GTP hydrolysis (the RGS proteins) or retarding GDP release (the GoLoco proteins). Regulators of G-protein signaling rapidly accelerate the GTP hydrolysis of Gα subunits by stabilizing the transition state; while GoLoco motifs act as GDIs (guanine nucleotide dissociation inhibitors), preventing GDP dissociation by adding a second arginine side-chain to the contacts made to the bound nucleotide.

In this application note, we describe the use of the BMG LABTECH's POLARstar® Omega to monitor changes in the intrinsic fluorescence of a highly-conserved tryptophan located in the switch II region of Gα subunits (Fig. 1, "SII"). The conformational change in SII decreases the exposure of the Trp residue to the aqueous environment, re-sulting in an increase in the quantum yield. One can quantify this event by measuring the increase in Gα protein fluorescence at 350 nm upon excitation at 280 nm. In this application note, we have optimized the assay by varying concentration of Gα, changing assay buffers, and shifting excitation and emission wavelengths.

Materials & Methods

All experiments were conducted on the POLARstar Omega plate reader at ambient temperature using Corning Black Polystyrene 96-well plates. $G\alpha_{i1}$ was purified exactly as previously described and diluted to 1 µM in assay buffer (unless otherwise noted) and plated at an initial volume of 187 µL/well. Experiments were conducted using a 280 ± 5 nm and 350 ± 5 nm filter for excitation and emission, respectively, unless specified otherwise.

To maximize data acquisition during the experiment, typical data collection was divided into three distinct phases – baseline (-15 - 0 s), activation (0 - 132 s), and plateau phase (132 - 158 s). Data were collected at 1, 0.6 and 2 s intervals for baseline, activation and plateau phases, respectively, using the fast kinetics (well mode) function on the Omega. At 0 s, 8 µL of 0.5 M NaF and 5 µL of 1.2 mM $AlCl_3$ were injected sequentially with a 5 s delay. NaF and AlCl3 undergo a chemical reaction to form AlF_4^-, which mimics the leaving phosphate group upon hydrolysis of GTP. This stable complex, $G\alpha_{i1}$ · GDP · AlF_4^-, mimics the active, GTP-bound state of $G\alpha_{i1}$. The gain was set to 50% relative to 200 µL of pre-activated $G\alpha_{i1}$ · GDP · AlF_4^- to avoid saturating the signal. The previously described GoLoco motif GDI peptide, AGS3Con, was used and shown to inhibit the formation of $G\alpha_{i1}$ · GDP · AlF_4^-.

Buffers

Phosphate assay buffer (pH 8.0) - 100 mM NaCl, 100 µM EDTA, 2 mM $MgCl_2$, 2 µM GDP, 20 mM K_2HPO_4/KH_2PO_4 pH 8.0

HEPES assay buffer (pH 8.0) - 100 mM NaCl, 100 µM EDTA, 2 mM $MgCl_2$, 2 µM GDP, 20 mM HEPES

Tris assay buffer (pH 8.0) - 100 mM NaCl, 100 µM EDTA, 2 mM $MgCl_2$, 2 µM GDP, 20 mM Tris

Instrument Settings

Fluorescence Intensity - Well Mode

Keep default settings except for the following:

No. of kinetic windows - 3

Baseline

No. of intervals - 15, No. of flashes - 10, Interval time - 1 sec

Activation

No. of intervals - 220, No. of flashes - 10, Interval time - 0.6 sec

Plateau

No. of intervals - 13, No. of flashes - 10, Interval time - 2 sec

Rev. 07/2009
Keywords: **GDP, GPCR, GTP, Signaling, Tryptophan**

Injection - use 320 µL/s and keep smart injection unchecked

Pump 1 inject 8 µL at start time 15 s (at t=0 in the graphs)

Pump 2 inject 5 µL at start time 20 s (at t =5s in the graphs)

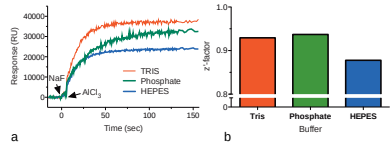

Fig. 3: (a) Signal intensities for 1 µM $G\alpha_{i1}$ in various assay buffers with common salts. (b) Z'-factors of the assay performed in various buffers.

Results & Discussion

In order to measure the effect of sample concentration on maximal response, we made serial dilutions of $G\alpha_{i1}$ from 3 µM to 50 nM in Tris pH 8.0 assay buffer. The most robust response was seen at the highest concentration of $G\alpha_{i1}$ tested (Fig. 2, red), but a change in fluorescence was detectable at all concentrations. To compare the quality of the signal for each concentration, a Z'-factor was computed for each concentration.

$$Z' = 1 - \frac{3\sigma_{plateau} + 3\sigma_{baseline}}{|\mu_{plateau} - \mu_{baseline}|}$$

This calculation accounts for the magnitude of the signal change upon excitation ($\mu_{plateau}$ - $\mu_{baseline}$) as well as the standard deviation of data collected during the plateau phase ($\sigma_{plateau}$) and baseline phase ($\sigma_{baseline}$). Using the Z'-factor, 3 µM of $G\alpha_{i1}$ was seen to have no advantage over 1 µM $G\alpha_{i1}$ (i.e., both Z'-factors > 0.9) while the quality of the data decreased at concentrations under 1 µM (not shown).

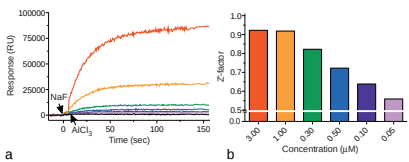

Fig. 2: (a) Signal intensities at varying concentrations of $G\alpha_{i1}$ as activated by aluminum tetrafluoride addition (sequential application of NaF and $AlCl_3$.) (b) Z'-factors of the assay at varying $G\alpha_{i1}$ concentrations.

To assess the effect of assay buffer composition on signal intensity, we measured the activation of 1 µM $G\alpha_{i1}$ in assay buffer prepared with various common buffer salts (Fig. 3a). The quality of the measurements, as determined by the Z'-factor, was similar for all of the buffers (Fig. 3b) although the maximum signal was observed with Tris assay buffer and the lowest magnitude was observed using HEPES assay buffer.

To verify that the assay is detecting the rate of Gα activation and is sensitive to changes in this rate, we incubated 500 nM of $G\alpha_{i1}$ with 5 µM AGS3Con peptide, a previously described GDI. As expected, the addition of AGS3Con (Fig. 4) dramatically dampened the maximal response of $G\alpha_{i1}$, as compared with 500 nM $G\alpha_{i1}$ alone.

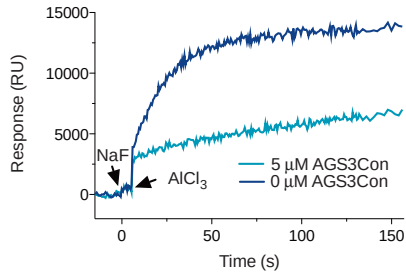

Fig. 4: Effect of 5 µM of the GDI peptide AGS3Con on intrinsic tryptophan signal intensity upon aluminum tetrafluoride activation of 500 nM $G\alpha_{i1}$.

Conclusion

In this application note, we described a robust automated assay system for measuring G-protein α subunit activity. The assay is a sensitive and high-quality means to measure G-protein activation without the use of radiolabeled nucleotides.

Performing the assays with the POLARstar Omega 96-well plate reader with on-board injectors offers the advantage of automating the assays in triplicate on multiple Gα mutants or multiple modulators of spontaneous GDP release.

Enzyme kinetic measurements for a combinatorial library of inhibitors of *Pseudomonas* elastase

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- ***Pseudomonas* elastase activity monitored using Abz-Nba internally quenched substrate**
- **K_m and V_{max} calculated using MARS Data Analysis Software**
- **Michaelis Menten kinetics determined for a library of 160 elastase inhibitors**

Introduction

Pseudomonas elastase (pseudolysin, LasB) is a metalloprotease virulence factor secreted by the opportunistic pathogen *Pseudomonas aeruginosa*. As one of the main virulence factors of this bacterium, it contributes to chronic and intractable infection in various disease states from the cystic fibrosis lung, to chronic ulcers of the skin.
The central role of LasB makes it a key drug target in this process, and so a library of inhibitor candidates was developed for screening against this enzyme. Assays were performed using a filter-based microplate reader from BMG LABTECH, which allowed highly adaptable data capture, and screening of multiple compounds in parallel. Data was analysed directly within the MARS software, which allowed extraction of subsets of data post-assay.

Assay Principle

The assay principle is shown in figure 1.

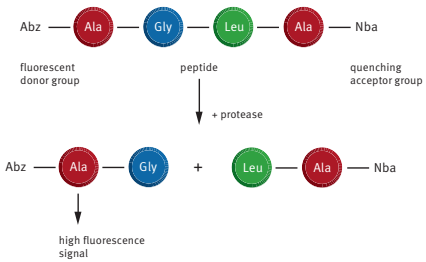

Fig. 1: Assay principle for the determination of LasB activity.

The internally quenched protease substrate Abz-peptide-Nba (2-aminobenzoyl-Ala-Gly-Leu-Ala-4-nitrobenzylamide) gives only a low fluorescence signal. After cleavage of the peptide bound by LasB the fluorescent donor group cannot transfer the energy to the quenching acceptor group resulting in a high fluorescence signal which is directly related to the enzymatic activity.

Materials & Methods

- Abz-Ala-Gly-Leu-Ala-Nba (Peptides International, US)
- Library of LasB inhibitors, synthesised at The School of Pharmacy, Queens' University, Belfast

Kinetic measurement
LasB was prepared at 1 in 1000 dilution from 100 µg/mL stock, and used at 10 µL per well, giving a working concentration of 1 ng of LasB per well.
The K_m of the substrate was first calculated by assay of a series of concentrations of substrate from 20 µM to 1000 µM, against a fixed concentration of LasB.

Inhibitor studies
Stock solutions of inhibitors were prepared in DMF at 10 mM, and further diluted when required. Instrument settings employed were as follows:
No. of flashes per well: 10
Target temperature: 37 °C
Ex filter: 310/10 nm and Em filter: 460/10 nm

All assays were performed in buffer containing 0.05 M TRIS HCL, 2.5 mM $CaCl_2$, 1 % DMF, pH 7.2, across a range of concentrations of inhibitor.

Results & Discussion

The results can be seen in figure 2, followed by graphical display of the rate of hydrolysis vs substrate concentration (figure 3), and a double reciprocal or Lineweaver-Burk plot, figure 4.

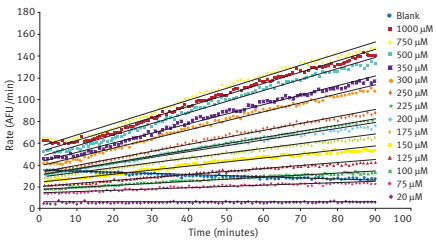

Fig. 2: K_m determination for the LasB substrate Abz-Ala-Gly-Leu-Ala-Nba.

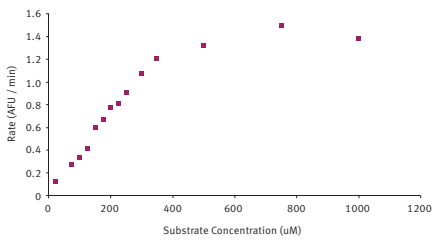

Fig. 3: Rate of substrate hydrolysis by LasB vs substrate concentration.

Rev. 10/2009
Keywords: **Elastase, Enzyme kinetics, Inhibitor screening, K_m, V_{max}**

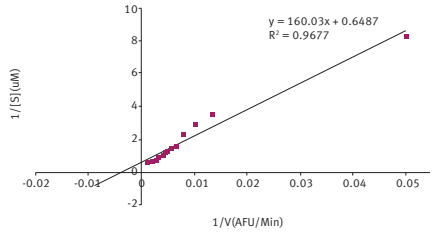

Fig. 4: K_m determined by Lineweaver-Burk plot. The double reciprocal of the data from figure 3 is used to linearise the data.

The slope of the line on the Lineweaver-Burk plot gives K_m / V_{max}, while the X- intercept gives - 1 / K_m, and the Y- intercept, 1 / V_{max}. The data from figure 4 can therefore be used to calculate K_m by solving the equation of the line Y = mX + c, where m = slope.

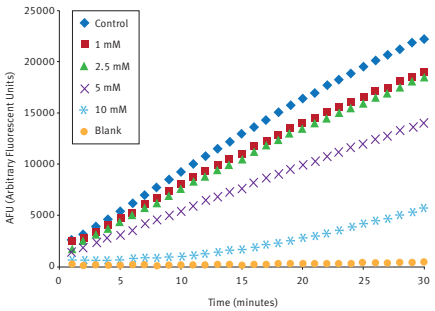

Fig. 5: Progress Curves for hydrolysis of substrate by LasB in the presence of a range of concentrations of a typical LasB inhibitor.

Linear transformation provides a value for the slope of the line, according to the equation y = mx + c. The K_i could be determined for each inhibitor in turn, via the Michael Menten equation (figure 6 and table 1).

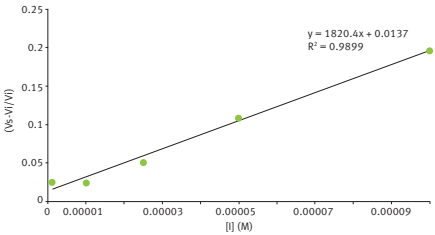

Fig. 6: Linear transformation of progress curves for a typical LasB inhibitor.

Table 1: K_i Values (µM) for inhibitor library. 'NI' (No Inhibition) has been stated for values over 1000 µM. Values in grey identify a general trend for low K_i values in inhibitors containing P'1 Trp and Tyr residues.

Ki (µM)								
	Basic		Aromatic		Large Aliphatic		Acidic	
P'_2	Lys	Arg	Phe	Trp	Val	Leu	Asp	Glu
P'_1								
His	332	(NI)	21	18	47	306	(NI)	(NI)
Arg	135	(NI)	224	125	(NI)	(NI)	650	(NI)
Lys	433	(NI)	126	(NI)	555	123	971	(NI)
Ile	190	(NI)	(NI)	366	1.8	1.3	142	(NI)
Phe	76	(NI)	146	206	11	645	(NI)	(NI)
Leu	14	623	113	300	(NI)	53	587	(NI)
Trp	10	25	1.1	49	4.1	3.7	38	91
Ala	153	115	(NI)	395	51	21	316	(NI)
Met	3.9	6.6	867	204	98	(NI)	7.0	(NI)
Pro	766	56	(NI)	562	157	246	(NI)	(NI)
Cys	274	646	131	108	161	(NI)	(NI)	(NI)
Asn	289	280	37	70	180	508	(NI)	(NI)
Val	22	69	72	(NI)	10	69	(NI)	(NI)
Gly	451	641	51	122	457	138	(NI)	(NI)
Ser	(NI)	444	75	(NI)	229	510	(NI)	(NI)
Gln	380	217	(NI)	91	937	540	(NI)	(NI)
Tyr	8.5	3.0	6.5	14	0.77	33	5.5	27

Conclusion

The microplate readers from BMG LABTECH offer convenient calculation of K_m, adaptable assay optimization, parallel assay of multiple inhibitors, and isolation of subsets of data post-assay.

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Analysis of migration using the Oris™ cell migration assay TriCoated kit

Iman van den Bout
Inositide Laboratory, Paterson Institute for Cancer Research, Manchester, UK

- **Knockdown U2OS cells were used with the Platypus® Oris™ Cell Migration system to detect if phosphoinositide metabolism is required for cell migration**
- **Real-time monitoring of cell migration using a CO_2 gas vented system**

Introduction

The integrin family of proteins are essential for the ability of cells to adhere and to migrate on extracellular matrix components.There are two distinctive groups of integrins within the family that can be identified by their ability to bind to RGD containing extracellular matrix components such as fibronectin and those that act as collagen receptors. The migration of epithelial cell sheets can play an important role during development or wound healing. Phosphoinositides such as PtdIns3,4,5*P3* and PtdIns4,5*P2* are thought to play a role in migration. To study migration in cell lines, scratch assays were typically performed where a confluent monolayer was scratched to remove a strip of cells and then the movement of the cells from the edges into the gap was quantified. This method has several drawbacks including the removal of the ECM components and the rupturing of cells which could all skew the results.
The Oris™ Cell Migration Assays specifically the TriCoated kits presents certain advantages over the traditional migration assay including the ability to test the effect of different ECM components on migration. With the use of siRNA and the Oris™ TriCoated Cell Migration Assay we show here that phosphoinositides are important for migration. We were able to measure migration on different ECM components in real-time using a BMG LABTECH microplate reader fitted with a CO_2 delivery system making it possible to keep cells in the plate reader for up to 48 hours.

Technology Overview

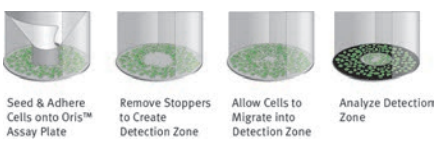

Fig. 1: Oris™ Cell Migration Assay system for Platypus Technologies.

The Oris™ Cell Migration Assay is designed with unique Oris™ Cell Seeding Stoppers, Detection Mask, and Removal Tool. Simply seed the cells in each well, allow them to adhere, remove the Stoppers to create a detection zone, and measure the migration. The Oris™ Cell Migration Assay - TriCoated offers three surfaces (32 wells ach of Tissue Culture Treated, Collagen I, and Fibronectin) on a single 96-well microplate. More detailed information can be found on the Platypus website. www.platypustech.com/discoverassay.html.

Materials & Methods

- DiI-C16 (Molecular Probes®, Invitrogen)
- siRNA (Dharmacon)
- Oris™ Cell Migration Assay (Platypus Technologies, LLC)
- Microplate reader (BMG LABTECH)

Cells were grown in DMEM obtained from Sigma with FCS, pen/strep and glutamate from Invitrogen. Phenol red-free DMEM was obtained from Gibco. The osteosarcoma cell line U2OS was used in these assays.

Migration assay
U2OS cells were seeded in 6 well plates at 100000 cells/well and transfected the next day with 10 µL 20 µM siRNA per well along with 3 µL DharmaFECT. Negative control siRNA was also transfected separately. After overnight incubation, cells were trypsinised, washed and counted. The total amount of cells needed for each assay was suspended in 1 mL phenol red-free medium containing 2.5 µM DiI and cells were incubated at 37°C for 30 minutes. Afterwards, cells were centrifuged and resuspended in phenol red-free medium to a concentration of 300000 cells/mL. Gel loading tips were used to deliver 100 µL of all suspension into each well of an Oris™ Cell Migration Assay TriCoated plate. For each condition one well without a Cell Seeding Stopper served as the positive control. The plate was then incubated overnight at 37°C. The following day the Cell Seeding Stoppers were removed from test wells and the medium was flicked out. Subsequently, 200 µL phenol red-free medium was added to each well. The Detection Mask was attached to the bottom of the plate and the plate was put in the pre-warmed plate reader.
For all experiments the fluorescence was measured with excitation at 544 nm and emission at 590 nm. Readings were taken at 20 minute intervals over a period of 48 hours using the bottom optic. The gain was adjusted as 70 % of the positive control.
After completion of the assay the data was analysed using the BMG LABTECH supplied MARS Data Analysis Software. First, a baseline correction was performed on the raw data using the data from the 6th to the 9th datapoints. Next, the curve was smoothed using moving averaging over three datapoints.

Results & Discussion

U2OS cells were transfected with a control non-targeting siRNA or with three different siRNA pools targeting specific proteins (A, B, C). Signal curves for tissue culture (TC) treated, Collagen I and Fibronectin coated wells are shown in Fig. 2, 3 and 4.

Rev. 11/2009
Keywords: **Bottom reading, Coating, Collagen, Fibronectin, Integrins, Migration**

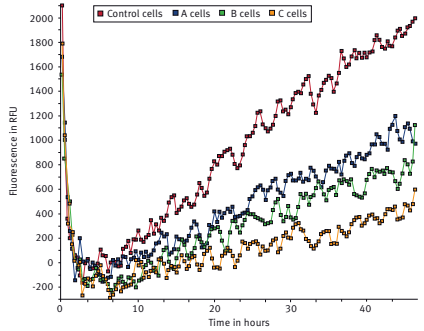

Fig. 2: Signal curves for cells on tissue culture treated surface. (n=6)

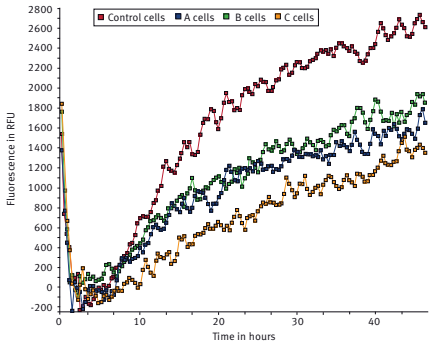

Fig. 3: Signal curves for cells on Collagen I coated surface. (n=6)

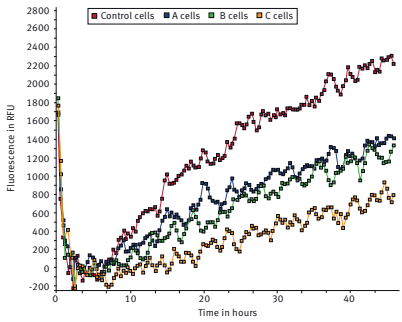

Fig. 4: Signal curves for cells on Fibronectin coated surface. (n=6)

The sum function in the kinetic calculation window was used to determine the area under the curve for each sample. The sum values were averaged and the population standard deviation was calculated (Fig. 5).

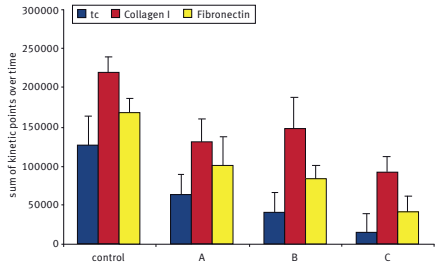

Fig. 5: Graph representing migration of knockdown cells on different surfaces.

The data indicates that U2OS cells transfected with control siRNA migrate the fastest on Collagen I while migration was somewhat reduced in both fibronectin and TC-treated wells. Migration dropped by 34% in cells transfected with siRNA pool A on TC-treated wells while the drop is 41% on Collagen I and 45% on Fibronectin compared to that of the Control conditions. Cells transfected with the siRNA B pool demonstrated a similar drop in migration to siRNA pool A across all surfaces tested. The knockdown of siRNA pool C had a more pronounced effect with migration dropping 75% on TC-treated wells, 53% on Collagen I and 69% on Fibronectin surfaces relative to the control. From this data it is clear that the C siRNA construct inhibits migration to a large extent in cells plated on TC-treated wells and on Fibronectin while there was a somewhat smaller effect in cells on Collagen I.

Conclusion

This assay shows that the knockdown of genes A, B, or C has an effect on cell migration. Migration is inhibited on Tissue Culture Treated, Fibronectin, and Collagen I surfaces suggesting that the effect of these genes is not integrin-specific. Since all three proteins knocked down in these cells are involved in phosphoinositide metabolism we can conclude that phosphoinositides are of importance for cell migration.

Cellular dopamine and intracellular calcium signaling using the next generation HTS microplate reader

Mark Koeppel[1] and E.J. Dell[2]
[1] Invitrogen [2] BMG LABTECH Inc.

- **Direct optic bottom reading, dual emission detection and high resolution well scanning are microplate reader features that greatly enhance all cell-based assays**
- **Fluo-4 Direct™ Calcium Assay and Tango™ Dopamine Receptor 1 assay**

Introduction

This application note highlights the direct optic bottom reading performance of the BMG LABTECH microplate readers in two different cellbased fluorescence intensity assays from Invitrogen. First, the intracellular calcium response to histamine was measured in HEK 293 cells using the Fluo-4 Direct™ Calcium Assay. Advanced high resolution cell layer scanning shows a correlation with the calcium signal and cell location in the well. Second, arrestin recruitment through activation of the dopamine D1 receptor with dopamine (D1 agonist) was measured using Tango™ D1-bla U2OS cells and a live cell FRET substrate (LiveBLAzer™). The fluorescent signal generated in the cells by the LiveBLAzer™ substrate greatly benefits from the PHERAstar® *FS´* unique bottom read dual emission detection. The PHERAstar *FS* is the next generation HTS microplate reader because it has many unique features not found on any other instrument. For this application note those unique features include: direct optic bottom reading; dual emission detection; injection at the point of measurement; high resolution well scanning (30x30 matrix); and orbital averaging.

Materials & Methods

- Black, clear bottom 384 well plates (Corning)
- Tango™ D1-bla U2OS DA Assay Kit (Invitrogen)
- Fluo-4 Direct™ Calcium Assay Kit (Invitrogen)
- LiveBLAzer™-FRET B/G Loading Kit (Invitrogen)
- HEK293 cells

Fluo-4 Direct™ Calcium Assay

HEK 293 cells were seeded into 384-well microplates at 15,000 cells/well in 25 ul of assay medium. An equal volume of 2X Fluo-4 Direct™ calcium reagent was then added to the well and the plate was incubated at 37°C in a CO_2 incubator for 60 min. Using the onboard reagent injectors, 5, 10 and 15 µl of histamine (270 nM) were injected into different wells and the intracellular calcium flux was recorded using the fast kinetic (well mode) setting on the microplate reader. Readings were taken every 0.5 sec for 90 sec and the histamine injection occurred at 10 sec.

The fluorescence signal was measured using the direct optic bottom reading and an assay specific Optic Module (EX/EM 485/520). After the calcium response was measured, a bottom reading, high resolution well scan of the cell layer was performed using a 10x10 scan matrix.

Tango™ D1-bla U2OS GPCR Cellular Assay

Tango™ D1-bla U2OS cells were seeded into 384-well microplates at 15,000 cells/well in 32 µl of assay medium. Dopamine, 8 µl of 5X concentration, (columns 1-10, 9 replicates each) was added to the cells to generate a dose response curve then incubated for 5 hr in a CO_2 incubator at 37°C. Following treatment, 8 µl of 6X LiveBLAzer ™ substrate was added for 2 hr at room temperature. Plates were read using direct optic bottom reading and a dual emission Optic Module that excites at 405 nm and emits at both 460 and 530 nm.

Results & Discussion

Fluo-4 Direct™ Calcium Assay

Figure 1 shows the intracellular calcium response measured using Invitrogen's Fluo-4 Direct™ Calcium Assay, in HEK 293 cells upon the injection of three different volumes (5, 10, and 15 µl) of histamine (270 nM). Different ranges can be defined to perform different calculations with those ranges. When the averages were calculated (inset graph Figure 1), some of the replicates were found to vary widely.

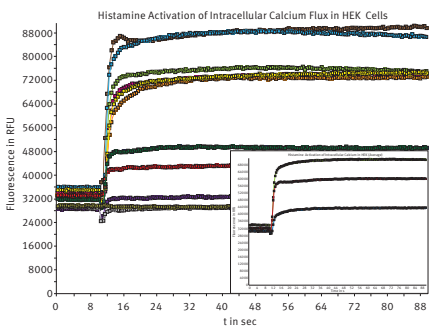

Fig. 1: Fluo-4 Direct™ intracellular calcium response in HEK 293 cells upon injection of histamine (5, 10 and 15 µL of 270 nM) in a 384 well microplate. Inset graph shows the average of four replicates.

After the kinetic measurement, advanced, high resolution cell layer well scanning was performed using direct optic bottom reading and a 10x10 matrix. As can be seen in Figure 2, the number of cells in the middle of each well varied, causing the signal to vary accordingly.

Rev. 02/2010
Keywords: **Bottom reading, Calcium, Dopamine, Fluo-4, GPCR, Injectors, LiveBLAzer, Well scanning**

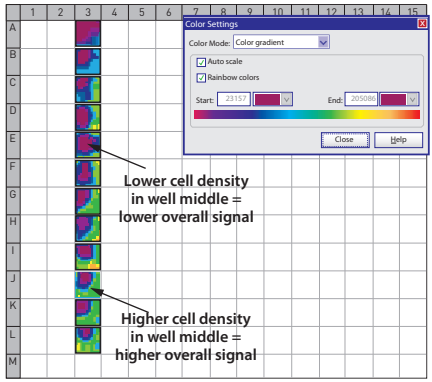

Fig. 2: Bottom reading high resolution cell layer scanning (10x10 matrix) shows the uneven distribution of HEK cells in each well. Samples with higher cell density in the middle of the well gave a more robust calcium response than lower density wells.

Well scanning can thus be used to help correct for cell number. To avoid such uneven cell distribution and results, three things can be done:

1) use pre-coated microplates that allow cells to adhere better;
2) decrease the injection speed so as not to disturb the cell layer; and
3) use the orbital averaging function, which measures and averages readings around the entire well.

Tango™ D1-bla U2OS Cellular Assay

The Tango™ division arrested U2OS cell line was used to measure the activity of the D1 receptor with dopamine through arrestin recruitment. The Tango™ GPCR assay uses beta-lactamase as a reporter to measure receptor activation. GPCR activation in the cell line recruits protease tagged arrestin to the activated GPCR which cleaves a non-native transcription factor tagged to the receptor's C-terminus. The released transcription factor enters the nucleus and stimulates the expression of beta-lactamase. Beta-lactamase is measured with the live cell fluorescent beta lactamase FRET substrate LiveBLAzer™. The substrate contains coumarin and fluorescein linked by the beta-lactamase substrate CCF2. In the non-active state, FRET occurs between the linked coumarin and fluorescein and emits a green signal measured at emission wavelength 530 nm. Upon receptor activation, beta-lactamase expressed in the cells cleaves the substrate resulting in an increased blue signal at 460 nm.

The ratio of blue to green cells is used to assess receptor activation and provides for a robust assay that is convenient for HTS. Figure 3 shows D1 receptor activation by increasing amounts of dopamine. As represented in the inset in figure 3, the cells change in color (green to blue) as increasing amounts of dopamine is added (columns 10 thru 1). A 4-parameter fit of the percent activation versus the log of the dopamine concentration gives an EC_{50} of 380 nM, which corresponds to the published 400 nM. Orbital averaging (2 mm), averaging multiple readings in an orbit in each well, allows for better Z' values (Table 1) and is recommended for cell based assays.

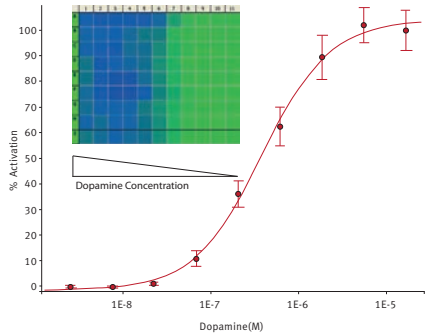

Fig. 3: Decreasing amounts of dopamine, a D1 agonist, (9 replicates) were added to Tango™ D1-bla U2OS cells (inset, columns 1-10) and the percent activation was plotted as a 4-parameter fit (negative control in column 11 was used as 0%).

Table 1: Dopamine Activation of Tango™ D1-bla U2OS Cells

	Z'	R^2	EC_{50}
With 2 mm Orbital Avg	0.76	0.998	380 nM*
Without 2 mm Orbital Avg	0.63	0.997	382 nM

*Corresponds with published results of 400 nM

Conclusion

Two cell-based signaling assays from Invitrogen, Fluo-4 Direct™ Calcium Assay and Tango™-bla U2OS GPCR Assay, were performed on a BMG LABTECH HTS instrument.

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A fast and simple method for measuring P-glycoprotein (Pgp) inhibition

Donald L. Melchior[1], Frances J. Sharom[2] and E.J. Dell[3]
[1] The Fluorosome Company, Worcester, USA [2] Department of Molecular and Cellular Biology, University of Guelph, Canada [3] BMG LABTECH, USA

- **The Fluorosome®-*trans*-pgp fluorescence intensity assay specifically measures the inhibition of P-glycoprotein multidrug transporter**
- **BMG LABTECH's microplate reader with onboard injectors used to set up fluorescence kinetic measurements**

Introduction

P-glycoprotein (Pgp; ABCB1), a member of the ATP-binding cassette (ABC) superfamily, exports structurally diverse hydrophobic compounds from the cell driven by ATP hydrolysis. Pgp expression has been linked to the efflux of chemotherapeutic drugs in human cancers leading to multidrug resistance. Pgp activity can also result in low oral absorption and poor brain penetration. Interaction of drugs with the Pgp may also cause an increase in toxicity of co-administered compounds.

Interaction of drugs with active transporters such as Pgp is of increasing interest to the pharmaceutical industry based, in large part, on new draft FDA guidelines requiring knowledge of whether a drug candidate is a substrate and/or inhibitor of Pgp.

The Fluorosome Company's Fluorosome®-*trans*-pgp assay, together with BMG LABTECH's microplate readers, provide a rapid, sensitive and specific reconstituted Pgp liposome assay system for identification of compounds that interact with the transporter. The assay measures the ability of a compound to compete with a Pgp substrate for transport, and determines the IC_{50} value. The reader's ability to inject at the point of measurement assures that no data is lost. Up to 50 readings per second taken concurrently upon injection, provide sample data for calculation of first order transport rates. Single concentration inhibition measurements can be made very rapidly, and an IC_{50} determination carried out in only 10 minutes thereby.

Assay Principle

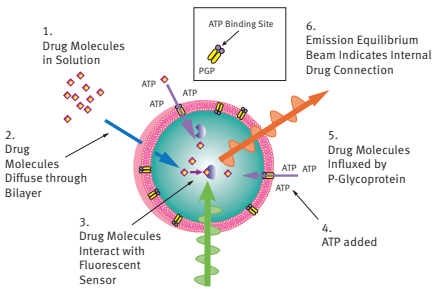

Fig. 1: Principle of the Fluorosome®-*trans*-pgp Assay.

Materials & Methods

The Pgp is a 170 kDa intrinsic membrane protein that effluxes a wide range of drugs from the cell. Pgp is also an outwardly directed flippase for fluorescent phospholipid and glycosphingolipid derivatives, which suggests that it may also translocate drug molecules from the inner to the outer membrane leaflet. The membrane lipid bilayer plays an important role in Pgp function and may regulate both binding and transport of drugs.

Isolation and Purification of Pgp

Purified Pgp (>90%) is isolated from Pgp-over-expressing CH^RB30 cells by detergent (CHAPS) extraction followed by affinity chromatography on Con A-Sepharose. Micellar Pgp is reconstituted into lipid bilayers by mixing in solution with CHAPS-solubilized micellar phospholipid, and the detergent is removed by gel exclusion chromatography. The resulting reconstituted Pgp is aliquoted and stored frozen at -70°C.

Manufacture of Fluorosome-*trans*-pgp

Frozen reconstituted Pgp is thawed and mixed in buffer with fluorophore (BSA-fluorescein) then converted into Fluorosome-*trans*-pgp by extrusion. The resulting unilamellar vesicles are separated from unencapsulated fluorophore by gel exclusion chromatography. The fluorosome-*trans*-pgp is then subjected to Quality Control Criteria; size, fluorescence, passive permeability, and ATP-stimulated transport of a control Pgp substrate.

The Fluorosome-*trans*-pgp assay procedure.

1. 98 µl of Fluorosome-trans-pgp in buffer containing 3 µM of the substrate S-HR was added to each well of a 96-well half-well plate.
2. A 2 µl aliquot of a DMSO solution of test compound was added. Each aliquot contained test compound at one of 7 concentrations to give, after dilution into the well, the desired concentration of compound. Blank, reference wells (no compound) had 2 µl of DMSO added to them.
3. The plate was placed in the microplate reader, and the fluorescence was monitored at 485/520 for 60 seconds as follows:
   a. a fluorescence baseline was established during the first 18 seconds;
   b. at 18 seconds, 5 µl of a solution of ATP in buffer was injected into each well (final concentration 2 mM), and the fluorescence was monitored for 42 seconds;
   c. The slope from 30 to 60 seconds was immediately calculated by BMG LABTECH's MARS data analysis software.

Rev. 04/2010
Keywords: **Flippase, Fluorescein, Inhibition dose response, Injectors, Multidrug transporter, P-glycoprotein Pgp**

A typical picture of fluorescence signals measured over time is given in Figure 2.

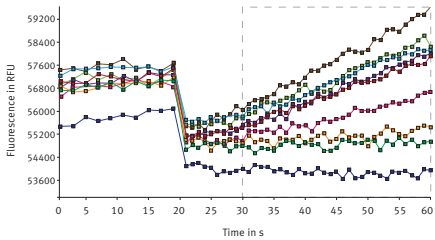

Fig. 2: Fluorescence kinetic signal curves for different samples.

Results & Discussion

The slope for each well (Fig. 2) was plotted against concentration of test compound, and IC_{50} values were calculated by using a robust fit for a single order decay (Fig. 3 and 4).

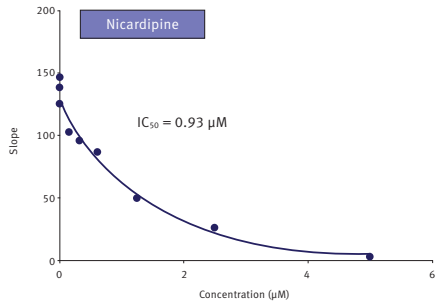

Fig. 3: Single order decay fit with Nicardipine as test compound.

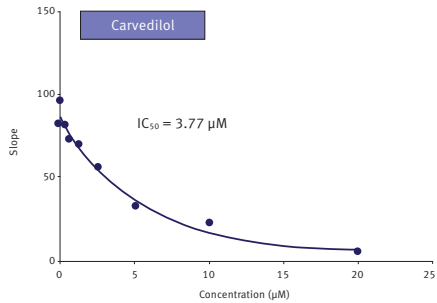

Fig. 4: Single order decay fit with Carvedilol as test compound.

Correlation of Fluorosome-*trans*-pgp IC_{50} Data and Cell Monolayer IC_{50} Data

IC_{50} values are given as µM concentrations. Bidirectional transport inhibition studies were conducted on the same set of compounds as were measured with the Fluorosome-*trans*-pgp assay, examples of which are shown in Fig. 5. Bidirectional transport inhibition studies employed LLC-MDR1 cell monolayers and [^{3}H] digoxin (0.1 µM) as the substrate.

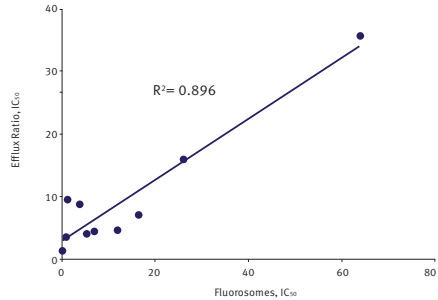

Fig. 5: Cell monolayer IC_{50} values reported as Efflux Ratio, i.e. changes in net transport Papp B-A - Papp A-B.

Results using the Fluorosome-*trans*-pgp assay are in excellent agreement with those obtained by cell culture methodology.

Conclusion

We report a novel assay specific for measuring inhibition of the P-glycoprotein multidrug transporter (Pgp; ABCB1) and an ideal instrument for its use.

- Simple assay procedure: No sterile conditions, non-compound specific, no radiolabel or LC-mass spectroscopy
- Specificity: Fluorosome®-*trans*-pgp contains only the P-glycoprotein transporter
- Low Sample Requirement: A 100 µl volume of Fluorosome®-*trans*-pgp Reagent per assay requires 1 nanomole drug
- Speed: 1 minute per inhibition assay - e.g. an 8 point IC_{50} determination + 2 references takes only 10 minutes
- Popular format: 96 well half-well or 384 well microplates - one well per inhibition assay

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Measure femtogram quantities of dsDNA

Ben Ferneyhough and Nick Parkinson
SBL UK, Abingdon, UK

- **Nucleic acid quantification was performed on a BMG LABTECH microplate reader in fluorescence mode**
- **Invitrogen's PicoGreen® assay was utilized to quantify dsDNA in 384-well HTS format**
- **Only 10 µL of sample is needed minimizing raw sample and waste**

Introduction

In the modern molecular biology laboratory, DNA samples are a precious commodity, where only small amounts can be sacrificed for quality control analysis, preserving the majority for downstream applications such as Next Generation Sequencing. These samples often contain very small amounts of DNA and so a technique has been developed to measure DNA to femtogram levels using low volumes (10 µL), utilising a high throughput well-plate format.
With the Quant-iT Picogreen reagent Invitrogen provides a solution for measuring low concentrations of DNA. This, together with Cambridge Biosciences AccuBlue enhancer, makes it a highly sensitive fluorescence assay for dsDNA detection.
This application note will investigate the use of very low volume DNA samples in a 384 well plate format on the BMG LABTECH filter-based microplate reader using the Picogreen reagent in combination with Cambridge Biosciences AccuBlue enhancer.

Materials & Methods

All materials were obtained through normal distribution channels from the manufacturers stated.

- Lambda DNA, Invitrogen
- Quant-iT Picogreen dsDNA reagent, Invitrogen
- 1x TE buffer, Promega
- AccuBlue High Sensitivity Enhancer 100x, Cambridge Bioscience
- black 384-well Optiplate-F, PerkinElmer
- Microplate Reader, BMG LABTECH

Consumables such as pipette tips and microcentrifuge tubes were used as required from various sources.

Preparation of the Picogreen working solution
The Picogreen reagent and the AccuBlue Enhancer are diluted in TE buffer to give a final dilution of 1:100 (Picogreen) and 1:10 (AccuBlue Enhancer). For example a 500 µL final volume of working solution contains 5 µL Picogreen reagent and 50 µL AccuBlue enhancer.

Preparation of the dsDNA dilution series
A 2fold serial dilution of the stock lambda DNA solution yielded eight concentrations from 100 pg/µL to 0.78 pg/µL in 1xTE.

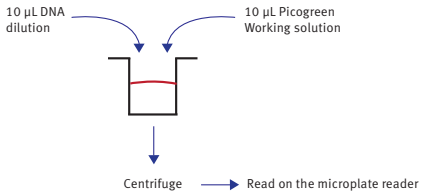

Fig. 1: Easy "Mix + Measure" protocol is used for dsDNA quantification.

Into a 384 well Optiplate, 10 µL of each DNA dilution was added in triplicate, followed by 10 µL of the Picogreen working solution resulting in a final DNA dilution series ranging from 50 pg/µL to 0.39 pg/µL per well. As a blank 10 µL TE buffer was added to 10 µL of Picogreen working solution.
The prepared 384 well plate was then centrifuged briefly at 1000xg to ensure that the solutions were at the base of the well. The plate was then placed into the microplate reader, a focus and gain adjustment was performed and the samples read using the following parameters.

Instrument settings

Excitation filter:	Ex485
Emission filter:	Em520
Gain:	was optimal adjusted
Focal height:	was optimal adjusted
Number of flashes:	10
Shake time:	30 seconds before reading cycle

The data produced was analysed using the BMG LABTECH MARS Data Analysis Software.

Results & Discussion

Figure 2 shows the dsDNA standard curve over the whole concentration range.

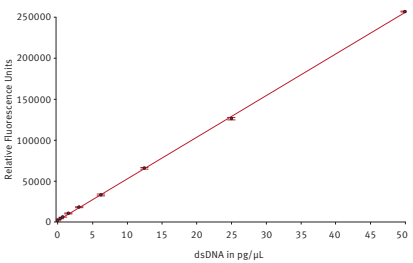

Fig. 2: dsDNA standard curve using DNA concentrations from 0.39-50 pg/µL.

Rev. 01/2011
Keywords: **AccuBlue, DNA quantification, Next Generation Sequencing, PicoGreen, Quant-iT**

The standard curve was linear over the whole range from 0.39 pg/µL to 50 pg/µL. Linear regression of the data yielded an R^2 value of 0.9998. Standard error bars between triplicate samples are shown.
After looking in more detail at the lower concentration range (Fig. 3), it can be seen that the calibration curve shows further linearity even into the femtogram per µL level.

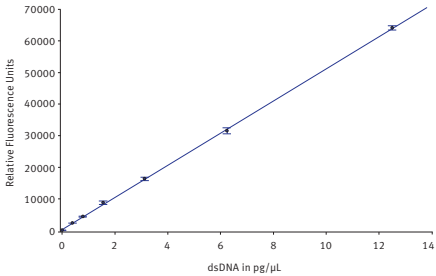

Fig. 3: dsDNA standard curve using DNA concentrations from 0.39-12.5 pg/µL.

Conclusion

The results presented here show that using the microplate reader from BMG LABTECH it is possible to reduce the volume of the sample to 10 µL and run the tests using a 384 well plate format. The method minimises waste, typically less than 5% of sample volume, with a fast, easy and accurate microvolume nuclear acid quantitation. This technique is used routinely in our laboratory.

Promotion of aggregation as a means of assessing the stability of antibody molecules

Alison Turner
UCB (Celltech), 216 Bath Road, Slough,SL1 4EN, UK

- **Stress can cause denaturation and aggregation of antibodies**
- **Aggregation propensity of antibody candidates was measured using a fluorescence based assay in 96-well format**

Introduction

During manufacture of antibody molecules, they are subjected to mechanical stress generated by processes such as pumping and filtration. This may cause denaturation and consequently aggregation due to exposure of the protein to air-liquid interfaces and shear forces, resulting in the ultimate loss in bioactivity.
Hence in the process of candidate selection, data regarding the aggregation propensity of antibody molecules in various buffer conditions would aid in the prediction of problems that would potentially be encountered during manufacturing and shelf-life stability. Shaking stress is a convenient method to screen and compare the robustness of antibodies in different buffer conditions in manufacturing and serves to mimic the effect of stress at air-liquid interfaces.
Experiments have been performed where various antibody molecules have been stressed by vortexing in 1.5 mL tubes and the extent of aggregation measured by the change in turbidity (absorbance at 340 nm/595 nm). This involves removing aliquots at various time points for manual analysis by a spectrophotometer and although this methodology is useful for screening different antibody candidates it cannot accommodate large numbers of samples and the data collection is inefficient.
A higher throughput (96 well plate format) screening method is required which would allow continuous real time measurement of aggregation within a convenient time window of analysis.
A filter-based microplate reader from BMG LABTECH equipped with shaking options (allowing for linear, orbital and double orbital continuous shaking) with both absorbance and fluorescence detection set up in the kinetic mode allowed us to explore a more efficient method for measuring aggregation propensity.
Initially transfer of the molecules and conditions used in the vortexing experiments to 96-well format proved unsuccessful resulting in negligible changes of aggregation promotion as judged by on-line absorbance analysis (340 nm/595 nm) even over a 7 daytime window.
However, the inclusion of a Teflon bead per well resulted in a change to the absorbance over a 24-48 h time window. The Teflon bead caused interference of the absorbance signal so the measurement of aggregation propensity was monitored by incorporation of an in situ fluorescent dye, Thioflavin T (a benzothiazole dye that exhibits enhanced fluorescence when bound to fibrous aggregates).

Materials & Methods

- Teflon beads (Polyballs Teflon 1/8" diameter, Polysiences)
- black 96-well microplate, Corning
- Filter-based microplate reader from BMG LABTECH

Experiment 1
The antibody molecule was used at 1 mg/mL final concentration in 50 mM sodium acetate/125 mM sodium chloride buffer (pH 5). A 2.5 mM Thioflavin T (ThT) stock solution was prepared in the buffer mentioned above.

The test solutions were combined as follows :
980 µL antibody solution + 20 µL buffer
980 µL antibody solution + 20 µL ThioflavinT solution
980 µL buffer + 20 µL ThioflavinT solution.

150 µL of test solutions were used per well of a 96-well microplate, in the presence or absence of one Teflon bead. A plate sealer was used to eliminate loss of sample by evaporation as the experiment was performed at 37°C and for extended times.

Instrument settings
No. of cycles: 44
Cycle time: 1810 sec
No. flashes per well: 10
Excitation: 440-10
Emission: 480-10
Shaking frequency (rpm): 1100
Shaking mode: orbital
Additional shaking time: 1800 sec after each cycle
Temperature: 37°C.

Experiment 2
Five different antibody molecules were diluted to 1 mg/mL final concentration in 50 mM sodium acetate/125 mM sodium chloride buffer (pH 5). All other solutions were prepared as mentioned in experiment 1. The instrument settings for screening five different antibody molecules were as described for experiment 1.

Results & Discussion

The first experiment was performed to establish whether inclusion of a Teflon bead could promote aggregation of an antibody molecule (Fig.1).

Rev. 02/2011
Keywords: **Aggregation, Denaturation, Fibril formation, Teflon beads, Thioflavin T**

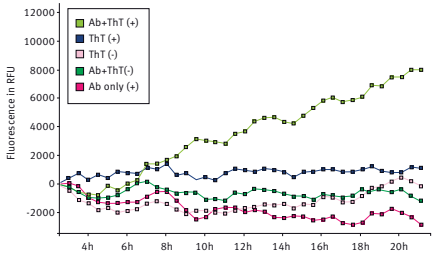

Fig. 1: Kinetic plot showing the effect of Teflon beads on aggregation promotion of an antibody; (+) indicates presence of bead ; (-) indicates the absence of bead. ThT=Thioflavin T. (Range set from cycle 4-40 to normalize for initial equilibration factors. Data presented as baseline corrected.)

There was only an increase in fluorescence intensity with time when the antibody sample was in the presence of the Teflon bead. It was noted that the overall fluorescence intensity was higher where Thioflavin T was present but there did not appear to be any aggregation promotion as a consequence of being present during the experiment.

In order to establish the effect of the Teflon bead and particularly in situ Thioflavin T, the antibody samples were analysed by Dynamic Light scattering (DLS), too (data not shown). The DLS data illustrated that there was only generation of large particles when the protein was in the presence of the Teflon bead. There was no particle formation when the protein was shaken in the absence of the bead and in situ Thioflavin T. Hence aggregation promotion was obtained as a consequence of shaking in the presence of the Teflon bead only and not by Thioflavin T.

A second experiment was done to screen different antibody molecules. The aim of this test was to investigate if it is possible to discriminate between 5 different antibody molecules with respect to aggregation propensity in a set buffer condition. The results are shown in Fig. 2.

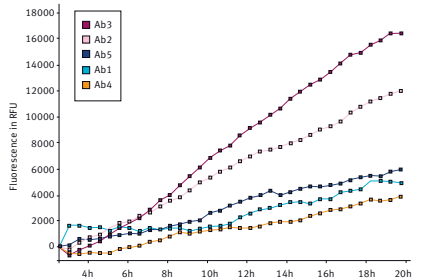

Fig. 2: Effect of shaking on the aggregation propensity of different antibody molecules in the presence of a Teflon bead (in situ ThT).

When five different antibody molecules were examined, it was possible to discriminate between them with respect to their different rates of aggregation in the buffer of interest. It was possible to calculate different rates of aggregation for each of the antibody molecules tested and to obtain a ranking order, see Figure 3. The ranking was in the order Ab3 > Ab2 > Ab5 > Ab1=Ab4, where A3 showed the greatest tendency to aggregate in the chosen buffer.

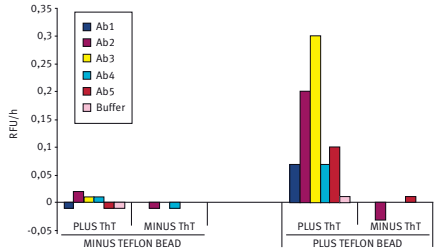

Fig. 3: Rate of Aggregation of the Different Antibody Molecules in the presence and absence of a Teflon bead.

Interestingly, the ranking order for the different antibody molecules was equivalent to that obtained when aggregation was promoted by an alternative method (agitation by vortexing - data not shown.)

Conclusion

The filter-based multidetection microplate reader from BMG LABTECH proved successful in preliminary experiments as a suitable high throughput screening method for the assessment of aggregation stability of different antibody molecules.

Since the mechanical stress using the shaking options of the microplate reader was more gentle compared with the vortexing approach in 1.5 mL tubes, the inclusion of a Teflon bead was required. This served to introduce more turbulence and hence increased the exposure to an air-liquid interface resulting in a faster and more efficient means of promoting aggregation.

Further studies are in progress to assess the use of different buffers and hence aid in pre-formulation work.

The ELISA*ONE*™ assay performed on a multimode microplate reader from BMG LABTECH

Miriam Uppill, Antony Sheehan and Ron Osmond
TGR BioSciences Pty Ltd.

- **ELISA*ONE*™ is a new technology from TGR BioSciences to measure ELISA in fluorescence mode**
- **ELISA*ONE*™ is both robust and sensitive as evidenced by the data presented for the detection of EGF, TNFα and IL-2.**

Introduction

ELISA*ONE*™ technology has been developed by TGR BioSciences, to provide a means of running high performance sandwich immunoassays in a user-friendly 96-well format. ELISA*ONE* assays use a traditional immuno-sandwich format, but with a major difference. The analyte and both antibodies are added to the ELISA*ONE* assay microplate at the same time, allowing solution-phase binding. After a short incubation period, unbound assay reagents and analytes are washed away, and only immunocomplexes containing both antibodies are detected. The whole process can take as little as 60 minutes to complete, and requires just a single wash step.

In contrast to other ELISA formats, the target-specific antibodies needed for the assay are not pre-bound to the microplate itself. The binding of antibodies to the analyte takes place in solution, allowing for efficient binding. This not only reduces assay times, but also affords several other benefits to the user. As the antibodies are not fixed to the plate, assays for several different targets can be performed in different wells on the same microplate, side-by-side. Another important benefit is reagent lifetime – many ELISA kits need to be discarded one month after opening, largely because the antibody-coated plates are often not stable after opening. With ELISA*ONE* however, the expensive assay reagents are in reusable bottles, and last a long time after the ELISA*ONE* microplate has expired. The inexpensive microplate can simply be discarded and another ordered, as they're all identical.

ELISA*ONE* assays for EGF, TNFα and IL-2 developed by TGR BioSciences, were used here to demonstrate the robustness and high sensitivity of the ELISA*ONE* technology.

Assay Principle

ELISA*ONE*™ - Protocol Overview

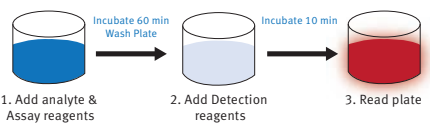

Fig. 1: ELISA*ONE* assay principle.

Materials & Methods

- Human EGF, IL-2 and TNFα were all from R&D Systems
- ELISA*ONE*™ microplates and assay reagents (Antibody Mix, Wash Solution and Substrate Mix) were from TGR BioSciences
- Filter-based microplate reader was from BMG LABTECH

Method:

ELISA*ONE*™ assays for EGF, TNFα and IL-2 developed by TGR BioSciences, were used here to demonstrate the robustness and high sensitivity of the ELISA*ONE* technology.

Briefly, analyte (50 µL) prepared in either BSA/PBS or RPMI containing 10% FBS depending on the experiment, was dispensed into duplicate wells of an ELISA*ONE* microplate. Antibody Mix (50 µL) – containing 2 antibodies specific for the target analyte – was added to the wells immediately after the analyte. The wells were sealed with a plate seal, and the ELISA*ONE* microplates were incubated for 1 hr at room temp, with gentle shaking. During this time the antibodies specifically bind to the analyte. Unbound components were aspirated from the wells, and the wells were washed manually with 3 x 200 µL of 1X Wash Solution. Substrate Mix (50 µL) was added to the wells, the microplate was covered with foil, and incubated for 10 min at room temp, with gentle shaking. The plates were uncovered, and the fluorescence signal (Ex 540 nm and Em 590 nm) was measured.

For recovery experiments, the amount of analyte in the well was calculated from a standard curve run on the same plate, prepared from 10-fold dilutions of analyte ranging from 1000 pg/mL to 0.1 pg/mL for EGF, and 10 ng/mL to 0.1 pg/mL for IL-2 and TNFα. The standard curve was fitted to a 4-parameter sigmoidal dose response equation, and the fitted equations were used to estimate the amounts of analyte recovered in each experiment.

Results & Discussion

1. Assay reproducibility and sensitivity

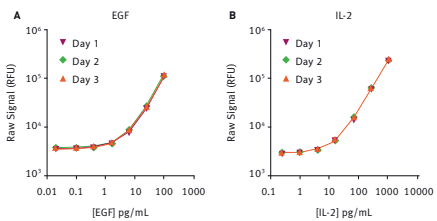

Fig. 2: ELISA*ONE* readouts depending on either EGF concentration (A) or IL-2 concentrations (B) measured at three different days.

Rev. 03/2011
Keywords: **EGF, ELISA*ONE*, IL-2, Immunoassay, TNFα**

Three assays were assessed for reproducibility over 3 separate experiments. Each day, either recombinant human EGF (Fig 2A), recombinant human IL-2 (Fig. 2B) or recombinant human TNFα (data not shown) were diluted to various concentrations in PBS containing 0.5% BSA in 96-well ELISA*ONE* assay plates (n=4 wells/concentration point). ELISA*ONE* was added to the wells, and the assays were incubated for one hour, washed, and incubated with ELISA*ONE* Substrate Mix for 10 min. The plates were read immediately using a POLARstar Omega microplate reader. The limit of detection (LOD) was approximated using the signal obtained with buffer-only controls + 3x standard deviations.

	Day 1	Day 2	Day 3
LOD (EGF)	0.1 pg/mL	0.4 pg/mL	0.4 pg/mL
LOD (IL-2)	1 pg/mL	4 pg/mL	1 pg/mL
LOD (TNFα)	4 pg/mL	1 pg/mL	1 pg/mL

2. Analyte recovery from tissue-culture supernatants

The assays ability to recover known amounts of analyte from tissue culture media was investigated.

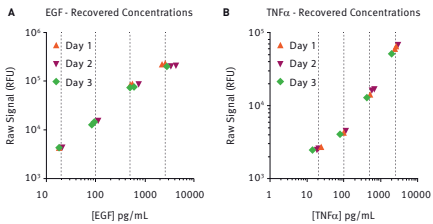

Fig. 3: Analyte recovery measurements for EGF (A) and TNFα (B).

Each day, either recombinant human EGF (Fig. 3A), recombinant human IL-2 (data not shown) or recombinant human TNFα (Fig. 3B) were diluted to concentrations representing more than a 3-Log range (20 pg/mL, 100 pg/mL, 500 pg/mL or 2500 pg/mL) in RPMI containing 10% FBS in 96-well ELISA*ONE* assay plates (n=2 wells/concentration point). ELISA*ONE* antibody mix was added to the wells, and the assays were incubated for one hour, washed, and incubated with ELISA*ONE* Substrate Mix for 10 min. The plates were read immediately. The assay was able to accurately recover analytes over a range of 3-logs difference in concentration.

The recovery was determined using an analyte-specific standard curve, diluted in PBS containing 10% FBS, and fitted to a non-linear 4-parameter sigmoidal curve. These calculations could be easily performed using the MARS evaluation software that interfaces with all BMG LABTECH microplate readers.

Conclusion

This application note establishes ELISA*ONE*™ (TGR BioSciences: www.tgrbio.com) as a robust and sensitive technology for the detection of biomarkers in biological samples. Its ease of implementation and substantially reduced preparation time, as compared to traditional ELISA assays, represents a substantial advance in ELISA technology. The filter-based microplate reader from BMG LABTECH provides an easy-to-use instrument that will measure this fluorescence based assay quickly, accurately and robustly.

Quantitative, high-throughput, fluorescent-based bioassay to detect Schistosoma viability

Emily Peak, Iain W. Chalmers and Karl F. Hoffmann
Aberystwyth University, UK

- **Conservative detection of pathogenic trematode Schistosoma involves low throughput microscopy**
- **Two fluorophores utilized as indicators of Schistosoma viability**
- **Dual chromatic fluorescence measurements performed on a filter-based microplate reader from BMG LABTECH**

Introduction

The blood fluke Schistosoma is a parasitic trematode that is responsible for more than 200,000 human deaths per year. The state of the art to detect Schistosoma viability involves microscopy and knowledge of parasite morphology. The lack of appropriate methods for quantifying Schistosoma viability blocks the development of new anthelmintics.
In this application note we present a fluorescence intensity-based microtiter plate assay to reproducibly detect schistosomal viability. The principle of the assay is based on differential membrane permeabilities of the dyes, fluorescein diacetate (FDA) and propidium iodide (PI). Fluorescein diacetate is able to cross the membrane of living cells. Once inside the cell, esterases will cut the diacetate and fluorescein is released resulting in a measurable fluorescence signal that is directly related to the number of living cells. In contrast, propidium iodide cannot enter a viable cell. This dye will only stain the DNA of dead cells when the membrane has been compromised. The simultaneous detection of both propidium iodide and fluorescein diacetate measures allowed us to develop a fluorescence-based, microplate bioassay to improve detection of schistosomal viability. Using this flexible bioassay, we demonstrate its versatility in detecting schistosomal viability in response to a known inhibitor of thioredoxin glutathione reductase (auranofin).

Materials & Methods

- Black-walled, clear and flat-bottomed 96-well plates from FisherScientific
- Black-walled, clear and flat-bottomed 384-well plates from Matrix

Schistosomula preparation and culturing
Schistosoma infected snails were exposed to light. The snails shed cercariae that were subsequently converted to schistosomula by mechanical transformation. After purification, microscopic examination was done to assess quantity and quality of purified schistosomula. After preparation the schistosomula were cultured in flasks for 24 hours and aliquoted into either 96-well plates (1000 parasites per 200 µL) or 384-well plates (200 parasites per 40 µL).

Schistosomula viability determination in response to auranofin
Purified schistosomula were cultured in microplates in phenol-red free DMEM for 24 h at 37°C and 5 % CO_2 in presence of varying concentrations (10-0.625 µM) of auranofin. After this all schistosomula were washed three times to remove test compound and culture media supplements. After washing the parasites, propidium iodide and fluorescein diacetate were simultaneously added to each well to obtain a final concentration of 2 µg/mL and 0.5 µg/mL respectively.

Measurement in the BMG LABTECH microplate reader
Samples were measured from the bottom in dual chromatic mode with 544 nm excitation/620 nm emission to detect PI and 485 nm excitation/520 nm emission to detect FDA.
All fluorescent values were obtained with the plate reader incubator set at 37°C to ensure efficient esterase conversion of fluorescein diacetate to fluorescein within live schistosomula. Inclusion of appropriate control samples (live and heat-killed dead schistosomula) compensates for any inter-plate variations.

Data Handling
Numbers of live and dead schistosomula in each well were calculated using the following equations:

$$\text{LIVE (FDA fluorescence)} = (F_{sample} - F_{neg.contr.}) / (F_{pos.contr.} - F_{neg.contr.})$$
$$\text{DEAD (PI fluorescence)} = (F_{sample} - F_{media\ control}) / (F_{neg.contr.} - F_{media\ control})$$

F = fluorescence intensity units
Samples = parasites incubated with test compounds
Negative control = parasites killed by heat shock
Positive control = untreated parasites
Media control = wells containing only media and no parasites

Schistosomula viability calculation:

$$\% \text{ Viability} = \frac{\text{Live (FDA fluorescence)}}{\text{Dead (PI fluorescence)} + \text{Live (FDA fluorescence)}} \times 100$$

Results & Discussion

With the help of a fluorescent microscope we could confirm that FDA is useful for the staining of living cells and that PI is useful for the staining of dead cells. We could also confirm that no co-localization of FDA- or PI-devided fluorescence was observed in the same cell of an individual schistosomula.

Optimal fluorophore/schistosomula incubation time
In pretests it was investigated how long both dyes should be incubated with the parasites in order to get maximal reproducible viability data. The results are shown in Fig. 1 and 2.

Rev. 03/2011
Keywords: **Cell death, Cell viability, Fluorescein diacetate, Propidium iodide, Schistosomula**

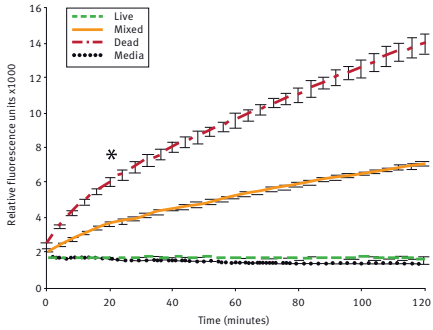

Fig. 1: Kinetic study of propidium iodide emission of schistosomula samples in 96-well format.

As expected dead parasites show the highest PI emission values whereas the signals for the live parasites are close to the media control and remain constant over time. The values for mixed samples containing live and dead schistosomula are inbetween. We chose an incubation time of 20 min to collect PI data as it provided an adequate time window to process multiple microtiter plates (* in Fig. 1). Whereas PI staining of dead schistosomula lead to an emission increase over at least 120 min, FDA staining showed different results (Fig. 2).

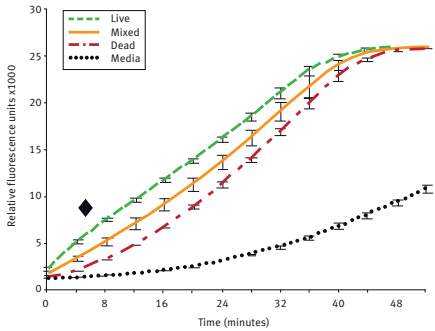

Fig. 2: Kinetic study of fluorescein diacetate emission of schistosomula samples in 96-well format.

Live, dead and mixed schistosomula populations generated emission data that quickly reached a plateau (51 min for 96-well plates). As expected the emission values for live schistosomula were highest, for dead lowest and for the mixed population we obtained intermediate values. The optimal FDA incubation time was calculated to be between 3 and 12 min. We chose 5 min to collect FDA data (♦ in Fig. 2).

Schistosomula viability in response to auranofin

To validate the assay, auranofin, an experimental TGR inhibitor and anti-schistosomula compound, was added to the parasites during parasite growth. There is a clear and titratable anti-schistosomula effect on schistosomula viability (Fig. 3)

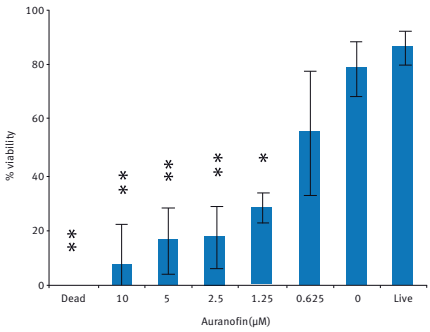

Fig. 3: Schistosomula viability in 96-well format calculated from fluorescent measurements after dual FDA and PI staining according to the formula explained in the materials and methods part.

Percent viability transformations into probit values also allowed an auranofin LD50 calculation (0.82 ± 0.49 µM). Maximum drug effect was seen at 10 µM auranofin, where microscopic examination of schistosomula confirmed that death was 100 %.

Conclusion

In this application note we demonstrate a quantitative, fast and inexpensive method to reproducibly measure schistosomula viability. The use of a dual staining method is important for counteracting any pipetting errors and for verifying that differences in fluorescence are genuinely caused by differences in mortality.

A fluorescence-based assay of the epigenetic enzyme Histonedeacetylase 1 (HDAC1)

Patricia Haus and Franz-Josef Meyer-Almes
University of Applied Science, Darmstadt, Germany

- **Histone modifications caused by HDAC are strongly related to epigenetic regulation of transcription**
- **HDAC inhibitors (HDIs) are a possible strategy in cancer therapy**
- **Fluorescent HDAC activity and inhibitor assay was performed on a microplate reader from BMG LABTECH**

Introduction

Post-translational histone modifications like acetylation play a pivotal role in the epigenetic regulation of transcription. Catalyzing the latter reaction HDACs affect various cellular processes especially cancerogenesis. Although the mechanism of starting cancerogenesis by epigenetic events is not clearly explained inhibition of HDACs has highlighted as a viable principle in cancer therapy. Inhibition of HDACs results in histone overacetylation that in turn can lead to a controlled cell death (apoptosis). Several HDAC inhibitors (HDIs) are in phase I or II clinical trials, for example suberoylanilide hydroxamic acid (SAHA; ZOLINZA®, Merck). In the last years research focused on the development of selective HDIs. To determine the inhibitory effect fluorogenic assays with recombinant proteins could offer a valuable performance. In this application note, a fluorescence microplate reader from BMG LABTECH was used to determine the inhibitor effect of SAHA against HDAC1.

Assay Principle

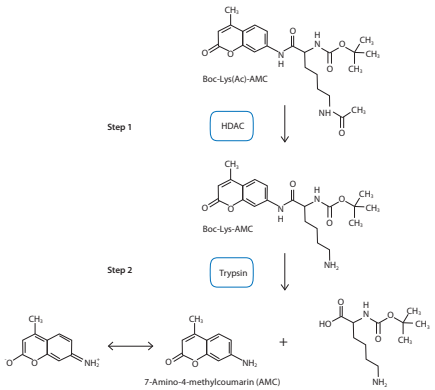

Fig. 1: Assay principle for HDAC activity evaluation.

Determination of HDAC1 activity was perfomed by a two-step enzyme assay. The principle bases on the ε-acetylated lysine moieties deacetylation of the substrate Boc-Lys(Ac)-AMC caused by HDAC. In a second step the deacetylated substrate is cleaved by trypsin resulting in the release of fluorogenic AMC (7-Amino-4-methylcoumarin; Fig.1).

Materials & Methods

- Microplate reader (BMG LABTECH, Ortenberg, Germany)
- Black 96-well half area plates (Greiner Bio-One)
- HDAC1 (BPS Bioscience)
- Boc-Lys(Ac)-AMC (Bachem)
- Trypsin from bovine pancreas (Sigma)
- SAHA (Cayman Chemical Company)
- FB188 buffer (15 mM Tris-HCl pH 8.0, 50 mM KH_2PO_4/K_2HPO_4, 250 mM NaCl, 250 µM EDTA and 0.001 % Pluronic F-68)

Evaluation of the K_M value of HDAC1

To determine the K_M value measurements using different substrate concentrations were carried out in FB188 buffer. In a first step HDAC1 (4.5 nM final concentration) was incubated with a 1:2 serial dilution of the substrate Boc-Lys(Ac)-AMC (initial substrate concentration was 512 µM). After a 1 hour incubation at 30°C trypsin (1.7 mg/mL) and SAHA (5 µM) were added in order to stop the reaction and to release the fluorogenic AMC (excitation filter: 340/10, emission filter: 460/10).

Evaluation of the IC_{50} value with SAHA versus HDAC1

Performing the assay as described above HDAC1 was incubated with SAHA for 15 min at room temperature after performing a 1:3 serial dilution (initial value 35 µM). Substrate was added in the second step with a final concentration of 20 µM followed by a incubation of 1 h at 30°C. Trypsin (1.7 mg/mL) and SAHA (5 µM) finished the reaction and AMC was measured directly.

Results & Discussion

The K_M value of HDAC1 was determined by using different substrate concentrations (Fig. 2).
Each data point corresponds to an extra kinetic measurement. The RFU (relative fluorescence unit) values are the average of the last 5 data points after the equilibrium of the enzymatic reaction is reached. The resulting K_M value was determined to be 58.89 µM.
Subsequent to the K_M evaluation the assay was performed in presence of a known HDAC1 inihbitor (SAHA). While the substrate concentration was strict at 10 µM the inhibitor were used in a range between 35 µM and 0.002 µM (Fig. 3).

Rev. 02/20t12
Keywords: **AMC, HDAC, Histone, Inhibitor screening, K_m**

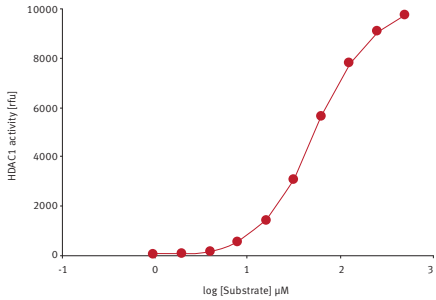

Fig. 2: K_M value of Boc-Lys(Ac)-AMC with HDAC1. HDAC1 (4.5 nM) was incubated with different concentrations of the substrate Boc-Lys(Ac)-AMC after performing a two-step activity assay by measuring the release of AMC (excitation filter: 340/10 nm; emission filter: 460/10 nm).

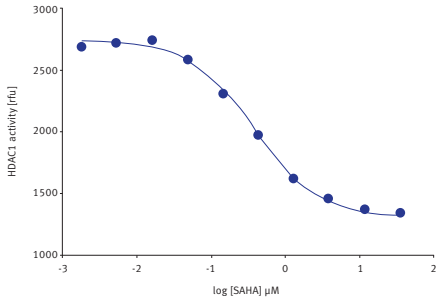

Fig. 3: IC_{50} value of HDAC1 with SAHA. HDAC1 (4.5 nM) was incubated with 1:3 serial dilution of SAHA and 20 µM Boc-Lys(Ac)-AMC after performing a two-step activity assay by measuring the release of AMC (excitation filter: 340/10 nm; emission filter: 460/10 nm).

The two-step activity assay using SAHA as inhibitor, results in a IC_{50} value of 374 nM.

Conclusion

Determining the activity of recombinant HDAC1 with a fluorogenic substrate using a microplate reader from BMG LABTECH offers high precision and performance. Using Boc-Lys(Ac)-AMC it has been shown that a wide range of substrate concentration results in a stable detectable signal. Furthermore, the assay allows for the determination of inhibitory effects against HDAC isoforms 1,2,3 and 6 as well as bacterial histone deacetylase-like amidohydrolase HDAH from *Bordetella/Alcaligenes* strain FB188.

Assessing pancreatic trypsin activity using the microplate reader from BMG LABTECH

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Liverpool NIHR Pancreas Biomedical Research Unit, Royal Liverpool University Hospital, University of Liverpool, UK

- **Trypsin activity as a measure for acute pancreatitis monitored using a fluorimetric kinetic assay**
- **Microplate reader increases efficiency and productivity compared to conventional spectrophotometer measurements**

Introduction

Pancreatic trypsin activity is one of the commonly used biomarkers for assessing severity of both in vivo and in vitro experimental acute pancreatitis. Currently, it is measured by fluorimetric assay using a spectrophotometer in which samples are excited at 380 nm and emissions collected at 440 nm. Although this method provides an accurate measurement of trypsin activity, it only allows testing of individual samples per time point (about 5 mins) and requires a considerable amount of trypsin substrate which is expensive. The BMG LABTECH microplate reader offers the possibility to test pancreatic trypsin activity with a minimal volume of sample and dyes in multiwell format and in real time.

Assay Principle

Trypsin is a serine protease and is known to cleave peptide chains mostly at the carboxyl side of lysine or arginine. This property is used to determine the trypsin activity in a fluorescent assay. The known trypsin substrate Boc-Gln-Ala-Arg-MCA contains after the amino acid Arg (arginine) a fluorescent dye called MCA (7-amino-4-methylcoumarin). After cleavage of substrate the MCA dye is set free and can be detected via fluorescence intensity. In this application note different ex/em settings were used to detect trypsin activity.

Materials & Methods

- normal and acute pancreas samples obtained from CD1 mice and C57BL/6 mice
- black 96w flat bottom microplates from Greiner
- Boc-Gln-Ala-Arg-MCA, Peptide, Osaka, Japan
- Microplate reader from BMG LABTECH

Preparation of pancreas samples

Mouse pancreata were homogenised by a motorised homogeniser on ice in tissue homogenisation buffer pH 6.5, containing (in mM) MOPS 5, sucrose 250 and magnesium sulphate. The resulting homogenates were centrifuged at 1500 g for 5 min, and 15 µL of each supernatant was added into a 96-well microplate.

Addition of trypsin substrate

Trypsin substrate Boc-Gln-Ala-Arg-MCA was added into assay buffer pH 8.0, containing (in mM) Tris 50, NaCl 150, $CaCl_2$ 1 and 0.1 mg/mL bovine serum albumin. The final working concentration for the substrate was 50 µM. The mixture (285 µL) was added into the microplate allowing the cleavage of trypsin by the substrate. The final volume in the well was 300 µL.

Detection of trypsin activity

The microplate reader was set in kinetic mode (plate mode). The samples were excited at 380 nm and emissions collected at 440 nm for a minimum of 5 mins. A standard curve was generated using commercially available porcine trypsin (Sigma) in different concentrations (0 – 150 µM). The fluorescence intensity at 3 mins for standards and samples was used for final analysis. The enzyme activity was calculated from the substrate conversion and related to the protein content in the well. To determine pancreatic protein concentration a BCA protein assay (Thermo, Rockford, USA) measuring absorbance at 562 nm was utilized.

Testing of different excitation and emission settings

The microplate reader was further used to test different excitation and emission settings for the determination of MCA. Next to the ex/em 380/440 combination an ex/em 355/440 combination was used and corresponding standard curves were obtained.

Results & Discussion

Detection of trypsin activity

For the porcine trypsin concentrations given, the microplate reader gave very accurate readouts (R2 = 0.9943) as shown in Fig.1. Pancreatic trypsin activity of samples from both normal and acute pancreatitis was accurately determined by the instrument. The results from the plate reader and the spectrophotometer (data not shown) are highly comparable (k = 0.9147).

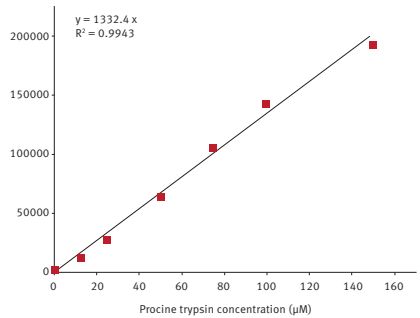

Fig. 1 Standard trypsin activity curve. Commercially available porcine trypsin was used as the trypsin standard in a concentration range of 0-150 µM.

Rev. 05/2012
Keywords: **AMC, Enzyme activity, Protease, Trypsin**

Testing several groups of samples using the spectrophotometer required a few hours of work but only required 10 minutes to finish the procedure on the plate reader, thus the instrument greatly increases efficiency and productivity. Moreover, only 1.5 µL of substrate was needed per sample in the microplate reader compared to 10 µL in the spectrophotometer.

Testing of different ex/em combinations

In the literature different excitation and emission wavelengths are used to determine the release of MCA in samples. Mostly the combination excitation/ emission 380/440 nm or 380/460 are described although the excitation maximum of MCA is at a shorter wavelength. Because of that an Ex355/440 filter combination was tried as well. Figure 2 shows signal curves obtained for these two different ex/em combinations under identical conditions.

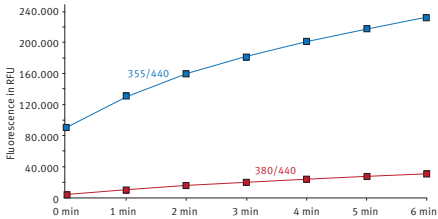

Fig. 2 Signal curves for 355/440 and 380/440 ex/em combination in a trypsin assay using Boc-Gln-Ala-Arg-MCA as substrate.

With the 355/440 ex/em combination higher relative fluorescence values are obtained compared to the conventional 380/440 ex/em wavelengths. But it is necessary to mention that also the blank value (RFU value at t = 0) is significantly higher. Based on these results a 355/440 could be preferred if only low signals are obtained.

Conclusion

Testing pancreatic trypsin activity in a BMG LABTECH microplate reader is quick and accurate. It allows for a much higher throughput of samples and has reduced the expense of costly consumables required for testing pancreatic trypsin in a spectrophotometer. Further, with this instrument it is very convenient to test different filter settings in a simple software-supported multichromatic mode.

Quantifying fluorescent ligand binding to GPCRs in live cells using the PHERAstar® *FS* - a new format for HTS

Alison Carter[1], Kevin Thompson[1] and Catherine Wark[2]
[1]CellAura Technologies Ltd, Nottingham, UK [2]BMG LABTECH, Aylesbury, BUCKS, UK

- **Fluorescent ligand binding to GPCRs in live cells measured using a PHERAstar® *FS***
- **Unlabelled competitor displacement IC_{50} curves quantified in 96-well format in < 3 minutes per plate**
- **A fast alternative to High Content Analysis for receptor binding and screening**

Introduction

Ligand binding affinities at G-protein coupled receptors (GPCRs) have historically been determined using a radioligand that competes for receptor binding sites against an unlabelled drug-like compound. However, the potential hazards of opensource radioisotope handling, and the environmental impact of radio-isotope disposal, make this a less desirable, costly technology. Development of fluorescent ligands for GPCRs provides a safer method for determining ligand binding affinities. However, quantification of fluorescent ligand binding tends to rely on high resolution fluorescence image capture. This approach can be lengthy and tedious, resulting in 96-well plate read times of approximately 30 minutes or more (depending on image number per well). Complex post-capture image analysis algorithms also extend data processing time. This results in High Content Analysis (HCA) assays that are too slow to be useful for High Throughput Screening (HTS).

In this study we use the adjustable focal height feature and advanced bottom reading of the PHERAstar *FS* plate reader from BMG LABTECH, in a live cell 96-well plate-based assay, to quantify fluorescent ligand binding associated with the adherent cell layer at the bottom of the well. Real-time, multi-spot fluorescence intensity measurements and averaging per well, incorporated with blank subtraction and sample displacement curve calculations were performed 'on the fly' as the wells were being analysed. Depending on the well scan area, number of lamp flashes, and number of spots per well analysed, this resulted in all-inclusive 96-well plate 'read to IC_{50} pKi curve plot' times of between 2.5 and 10 minutes. This is in stark contrast to HCA assays, which can take around 60 minutes to achieve the same endpoint.

In this study we illustrate the capability of the PHERAstar *FS* to perform fluorescent ligand binding assays using two of CellAura's fluorescent ligands for adenosine and dopamine receptors. Furthermore, we postulate the suitability of such a combination of plate reader and fluorescent ligand for higher throughput receptor binding assays and screening.

Materials & Methods

The two fluorescent ligands used in these experiments were developed by CellAura Technologies Ltd. Ligand CA200645 is an adenosine A3 selective ligand, while CA200767 is a dopamine D1 selective ligand. Both ligands are labelled with the BODIPY 630/650 fluorophore that fluoresces at the far red end of the spectrum.

The A1-selective antagonist DPCPX and the A3-selective antagonist MRS1220 were obtained from Tocris. The D1-selective antagonist, SCH23390 was obtained from Sigma-Aldrich. Black-sided, clear-bottomed, 96-well view plates were obtained from Greiner Bio-One.

The PHERAstar *FS* was fitted with an optic module for BODIPY 630/650 to provide Excitation at 620 nm and measuring Emission at 660 nm.

Experimental

CHO cells expressing human adenosine A1 or A3 receptors, or dopamine D1 receptors, were grown to confluence in T75 flasks, harvested, and seeded into 96-well black view plates and incubated at 37°C, 5% CO_2 for 24 hours prior to assay. On the day of assay, culture medium was aspirated and replaced with 100 µL serum-free media with or without an appropriate unlabelled competitor at 1.2x final concentration. Cells were incubated for 30 minutes at 37°C, 5% CO_2. Fluorescent ligand at 6x final concentration was added in a 20 µL volume (at a 1:6 dilution) to the appropriate wells. Cells were incubated for a further 30 minutes at 37°C, 5% CO_2. Cells were washed twice in HBS to remove unbound ligand, with a final 100 µL volume of HBS added to all wells.

Plates were read on the PHERAstar *FS* in bottom read mode using the 620/660 nm optic module. A 5x5 spot read matrix was selected to read from a 4 mm diameter in each well (a reduced diameter eliminates the edge of the well and any anomalies in the distribution of the cells in this region). Prior to reading the plate a Z-height scan was performed to identify the optimal height for quantifying fluorescence bound to the cell layer at the bottom of the well. An automated gain adjustment was also performed, to give an optimal gain of 3300. With the above settings applied to the measurement a read time of 2.5 minutes per plate was possible.

Results & Discussion

The BODIPY 630/650-labelled fluorescent ligands bound to their target receptors and were detected using the PHERAstar *FS*. Mean fluorescence intensity differences between positive controls (fluorescent ligand without unlabelled competitor) and negative controls (without fluorescent ligand) were used to calculate assay robustness Z' Factors. Binding of the fluorescent ligands was displaced by the appropriate unlabelled competitor. The pKi for the ligand-receptor combinations are given below.

The XAC-derivative adenosine A3 receptor antagonist, CA200645, binding to human adenosine A3 receptors

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Keywords: **Adenosine, Bodipy 630/650, Bottom reading, Dopamine, GPCR, Protein-ligand interaction, Receptor**

gave a Z'-Factor of 0.62 ± 0.07 (mean ± SEM, n=6). The adenosine A3-selective antagonist MRS1220 displaced the fluorescent ligand, CA200645, with a pKi of 8.58 ± 0.12 (n=5) (Fig. 1). This value is similar to the reported pKi for MRS1220 at the adenosine A3 receptor of 8.76, determined in a radioligand binding assay.

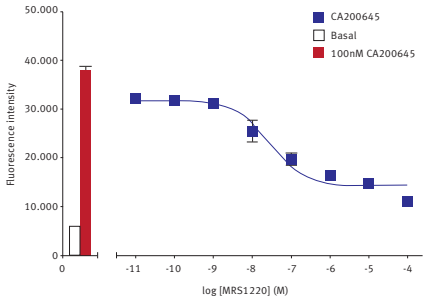

Fig. 1: Fluorescent ligand binding at human adenosine A3 receptors, and displacement with the unlabelled A3 selective competitor MRS1220.

The A3 receptor antagonist, CA200645, bound with lower affinity to adenosine A1 receptors, giving a Z'-Factor of 0.61 ± 0.1 (n=5). The A1-selective antagonist DPCPX was able to displace the A3-selective fluorescent ligand, to give a pKi of 7.47 ± 0.10 (n=4) (Fig. 2). The reported pKi for DPCPX at adenosine A1 receptors is 8.41.

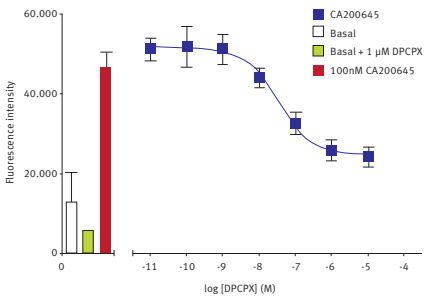

Fig. 2: Fluorescent ligand binding at human adenosine A1 receptors, and displacement with the unlabelled A1-selective competitor DPCPX.

The SKF-83566-derivative dopamine D1 receptor antagonist, CA200767, gave a Z'-Factor of 0.40 ± 0.13 (n=3). The D1-selective antagonist SCH23390 displaced the fluorescent D1 ligand to give a pKi of 8.63 ± 0.03 (n=3) (Fig. 3). The reported pKi for this ligand at the dopamine D1 receptor is between 9.3 and 9.7.

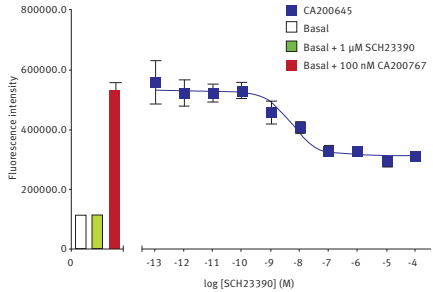

Fig. 3: Fluorescent ligand binding at human dopamine D1 receptors, and displacement with the unlabelled D1-selective competitor SCH23390.

Conclusion

These data illustrate the capability of the PHERAstar *FS* to rapidly and robustly quantify fluorescent ligand binding to live cells expressing recombinant human GPCRs in 96-well format using a simple 'add > mix > wash > read' assay protocol, analogous to whole-cell radioligand binding assays, but with inherent safety and cost advantages. It also offers considerable read and analysis time saving advantages over HCA assays, potentially making this format suitable for high throughput screening (HTS). In addition to the data shown CellAura have also used the PHERAstar *FS* to multiplex their BODIPY 630/650 ligand binding assay with other fluorophores such as Hoechst or DAPI to normalise for cell number. It may also prove possible to measure down-stream second messenger activation, such as Ca++ using the same cells, to add even more value to the assay data that can be achieved.

PHERAstar® *FSX*
PHERAstar® *FS*

Real-time quaking induced conversion assay for prion seeding

Maggie Nakamura
BMG LABTECH

- **Faster, higher throughput assay for prion seeding monitoring**
- **Advanced microplate reader functions for shaking and temperature control are key for assay effectiveness**
- **Fluorescence bottom reading measurements taken every 15 minutes for 20-68 hours**

Introduction

Prions are transmittable pathogens that cause an abnormal folding of a brain protein in both humans and animals. Infection results in brain damage and is fatal. Some examples of these neurodegenerative diseases are Scrapie, Bovine Spongiform Encephalopathy, and Creutzfeldt-Jakob Disease.

Previously, prions were studied using lengthy bioassays where infected animals were studied over long periods of time (1-6 months). This was both time consuming and costly to maintain the infected animal. Researchers at Rocky Mountain Laboratories in Hamilton, Montana have developed a new prion seeding assay called Real-Time Quaking Induced Conversion Assay (RT-QuIC) that gives end point quantitation for measuring the levels of prions in infected samples. This assay is both faster and a higher throughput compared to previous methods. The assay can be completed in as short as 20 hours and is as sensitive, if not more so, than whole animal models.
BMG LABTECH's Omega series of readers have the ability to shake and incubate microplates over long periods of time. A POLARstar® Omega was used to measure RT-QuIC samples every 15 minutes for 20-68 hours while alternately shaking for a minute and resting for a minute. A software script was developed to control the periods of shaking and resting needed to activate the assay.

Assay Principle

Combining parts of the original Quaking Induced Conversion (QuIC) assay and the amyloid seeding assay (ASA), the RT-QuIC assay is used to estimate the relative amount of prion seeding. The assay measures serial dilutions of samples, statistically estimating the seeding dose (SD). In the assay, very small amounts of infectious prions are added to normal prion protein to seed or cause the misfolding of the prion proteins as seen in the disease. The assay is quantitated by measuring serial dilutions of the samples and determining the loss of seeding activity (SD_{50}), which is the end point dilution.

Materials & Methods

- Nalgene Nunc black with clear flat bottom 96 well plate
- Nalgene Nunc plate sealer film
- POLARstar or FLUOstar® Omega, BMG LABTECH

Experimental
The instrument was set to incubate at 42°C for the entire 20-68 hour period. A script was created to use two test run protocols. One protocol was set to shake the plate for one minute and rest for one minute. The second protocol was set to take a fluorescence endpoint measurement using the bottom optic every 15 minutes. The fluorescent dye thioflavin T (ThT) is used as a prion seeding marker. When ThT is added to recombinant prion proteins, it becomes incorporated when polymerization occurs causing an increase in fluorescence over time.

Instrument settings
Fluorescence endpoint- Bottom Reading

Excitation filter:	450nm
Emission filter:	480nm
Flashes:	20
Gain:	2000
Shaking:	700RPM Double Orbital

Analysis
A Spearman-Kärber analysis, analogous to a bioassay's lethal dose of 50% (LD_{50}), was used to estimate a seeding dose or dilution at which 50% of the wells became ThT positive per gram of tissue (SD_{50}/g). For Spearman-Kärber analysis a dilution series with at least one dilution giving 100% ThT positivity and at least one dilution giving 0% ThT positivity was chosen.

Results & Discussion

A software script was used to measure RT-QuIC assays for 20-68 hours. Data was collected as an average of relative fluorescence units over 8 replicate wells. Only data for every 45 minutes is shown in each figure.
Both the FLUOstar and POLARstar Omega are able to shake in a unique double orbital pattern in addition to linear and orbital. For incubation the temperature was maintained at 42°C over the entire measurement period. The high quality design and manufacturing of the Omegas allow researchers to measure continuously for days at a time without a break in shaking or temperature fluctuations. Data was collected without interruption and outputted to BMG LABTECH's data analysis software (MARS) and Excel.

Rev. 12/2012
Keywords: **Aggregation, Bottom reading, Creutzfeld-Jakob-Disease, Prion, RTQuiC, Thioflavin T**

Figures 1 and 2, show the RT-QuIC assay as it relates to hamster scrapie brain homogenates (BHs). Figure 1 used hamster BHs that were harvested 80-85 days post inoculation (DPI), whereas figure 2 used hamster BHs that were harvested 10 days post inoculation.

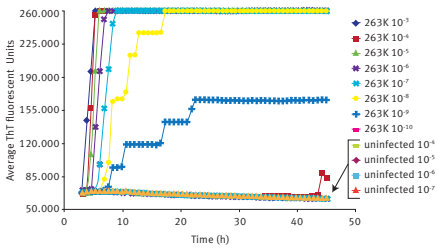

Fig. 1: RT-QuIC sensitivity: analysis of dilutions of a scrapie hamster brain homogenate stock 263K.

The 50% seeding dose (SD_{50}) is defined as the amount giving sufficiently enhanced ThT fluorescence in half of the replicate wells. In this case, the approximate SD_{50} was achieved with a 2 µl aliquot (the seed volume) of a 10E-9 dilution of the scrapie BH stock (dark blue line). Thus the Spearman-Kärber estimate for the SD_{50}/2µL is 10E9.1 and for the SD50/g is 10E12. The log of this SD_{50} (~11) corresponded well with the log of the LD50 (~10) found in the bioassay.

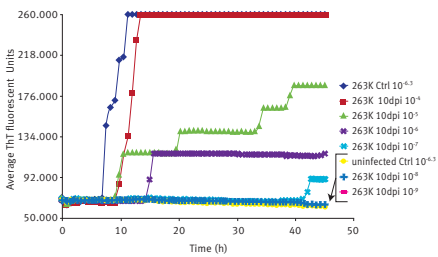

Fig. 2: RT-QuIC end-point dilution analysis of three 263K-inoculated preclinical 10 days post injection hamster BHs.

In this case, the approximate SD_{50} was achieved with a 2 µl aliquot (the seed volume) of a 10E–5 dilution of the scrapie 10dpi 263K BH stock (green line). This gave an SD_{50}/2µL of 10E5.5 and an SD_{50}/g of 10E8.2.
As expected, the SD_{50}/gram of tissue for the 85 DPI samples (10E12) was higher than the 10 DPI (10E8.2) because it had a longer time for onset. These RT-QuIC results correlated well with bioassay results. This data shows that the RT-QuIC assay could be considered a faster, higher throughput alternative to whole animal bioassays. This also shows that animals as early as ten days post inoculation can be examined using this assay.

Conclusion

Prion seeding can be measured faster and in a higher throughput using the RT-QuIC assay and a microplate reader. Some of the transmissible spongiform encephalopathies that have been shown to work using RT-QuIC include hamster and sheep scrapie, deer chronic wasting disease, Creutzfeldt-Jakob Disease (CJD), and Bovine Spongiform Encephalopathy (BSE). This assay is both faster and more sensitive than past bioassays. The Omega series of plate readers from BMG LABTECH are both functional and robust to withstand the many days of shaking at high speeds required for this assay.

Omega Series

Real-time fluorescence assay for monitoring transglutaminase activity

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Matrix Biology & Tissue Repair Research Unit, School of Dentistry, Cardiff Universit

- **Isopeptidase assay for kinetic analysis of transglutaminase activity**
- **Optimized protocols allow for rapid implementation and standardization of measurements**
- **Assay is amenable to high throughput analysis of regulators/inhibitors of catalysis**

Introduction

Transglutaminases (TGs) form a family of enzymes that catalyze various posttranslational protein modifications such as crosslinking, esterification and deamidation in a Ca^{2+}-dependent manner. Their main function is the formation of covalent Nε-(g-glutamyl)lysine bonds within or between polypeptides to stabilize protein assemblies. The activity of these enzymes is crucial for tissue homeostasis and function in a number of organ systems, and the lack of or the excessive crosslinking activity have been linked to human disease processes.
Here we perform kinetic measurements using recombinant TG2 and a fluorescent peptide model substrate in a format suitable for high-throughput analysis. This assay principle can be applied to kinetic studies on closely related enzymes including TG6 and can be optimised by modification of the backbone peptide sequence.

Assay Principle

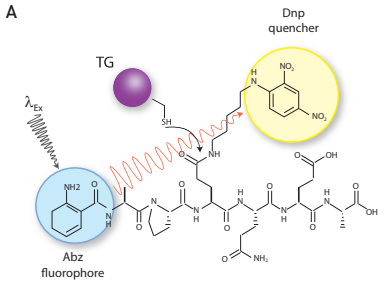

Fig. 1A: Structure of quenched substrate Abz-APE(γ-cad-Dnp)QEA whereby 2-aminobenzoyl (Abz) and 2,4-dinitrophenyl (Dnp) are fluorescent donor and quenching acceptor group, respectively.

The TG enzymatic reaction is a two-step process. The thioester intermediate of the enzyme formed with the substrate in the first step subsequently reacts with a nucleophile to regenerate active enzyme and release a 'crosslinked' polypeptide. The second step is reversible and, in the presence of an excess of crosslinked substrate, TG catalyzes isopeptide bond hydrolysis. We have exploited this latter activity for real-time monitoring of TG activity and characterize the effect of potential regulators on TG activity.

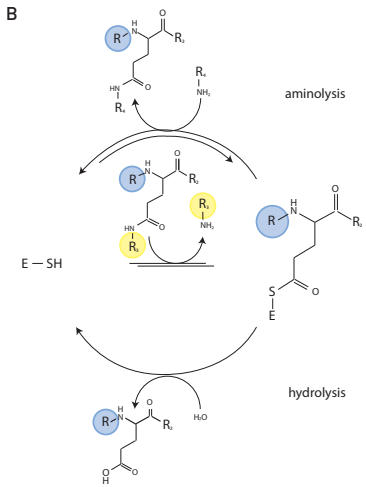

Fig. 1B: Reaction scheme for TG catalyzed isopeptidase reaction with substrate Abz-APE(γ-cad-Dnp)QEA in the presence of an excess of unlabeled primary amine (R_4-NH_2, e.g. glycine methylester). R: Abz-AP; R_2: QEA; R_3: $(CH_2)_4$-NH-Dnp; E-SH (TG2 with active site thiol group).

Abz-APE(γ-cad-Dnp)QEA is a quenched fluorescent probe derived from a known glutamine donor substrate that mimics a crosslinked TG reaction product. In this peptide the fluorophore (2-aminobenzoyl (Abz)) is quenched by a 2,4-dinitrophenyl-cadaverine (cad-Dnp) substituent on the first Gln residue, essentially replacing the Lys side chain in Nε-(γ-glutamyl)lysine linked peptides (Fig. 1A). TG2-catalysed hydrolysis of the isopeptide bond releases the cad-Dnp moiety (Fig. 1B) and consequently generates an increase in light emission at λ_{max}=418 nm from the Abz group. The thioester enzyme intermediate formed is subsequently deacylated through either aminolysis or hydrolysis. Specificity of the reaction is guided by the amino acid residues that surround the reactive Gln residue.

Materials & Methods

- Microplate reader from BMG LABTECH
- Black optical bottom 96-well plates (Nunc)
- Abz-APE(γ-cad-Dnp)QEA TG2 substrate (Zedira). 50 mM stock in DMSO
- Transglutaminase 2, 1 mg/ml stock, from Zedira

Assay buffer
The assay buffer consists of 62.5 mM Tris/HCl, pH 7.4, 125 mM NaCl. Add glycine methylester (or alternative

Rev. 04/2013
Keywords: **Enzyme activity, Kinetic studies, Posttranslational modification, Real-time, Transglutaminase**

amine donor substrate) and adjust pH immediately before use (at 37°C). Include DTT to prevent oxidative inactivation of TG.

Test protocol

Prime the microplate reader injectors with 20 mM $CaCl_2$ for enzyme activation (inj. 1) and H_2O or 20 mM $MgCl_2$ for control reaction (inj. 2). Pre-warm assay buffer and plate to 37°C and equilibrate instrument chamber at 37°C. Dilute substrate Abz-APE(γ-cad-Dnp)QEA (1:800) in assay buffer and add 80 µl of mixture into wells of the 96-well plate. Add desired amount of enzyme, e.g. 1 mg of TG2, and make up volume with H_2O to 90 µl. Transfer plate immediately into the reader and start program.

Reaction mixture (final concentrations)

The final assay volume is 100 µl and consists of 1-100 µg/ml TG2, 50 µM Abz-APE(γ-cad-Dnp)QEA, 10-55 mM glycine methylester or alternative nucleophile, as well as 1-5 mM DTT. After injection there is 2 mM $CaCl_2$ present in samples.

Instrument settings

Mode:	Fluorescence Intensity, plate mode
Filters:	Excitation: Ex320
	Emission: 440-10
Optics:	top
No. of flashes:	20
Cycles:	90
Cycle time:	40 s (for 12 wells)
Injection cycle:	10
Injection volume:	10 µL
Shaking:	5 s after each cycle
Temperature:	37°C

Results & Discussion

After Ca^{2+} injection an increase in fluorescence can be observed dependent on the concentration of enzyme in sample (Fig. 2).

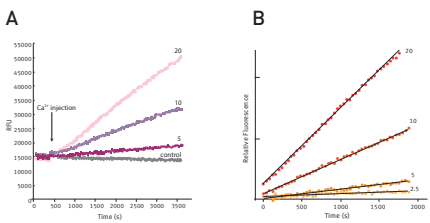

Fig. 2: Raw data (A) and processed data (B) of substrate Abz-APE (γ-cad-Dnp) QEA conversion at different concentrations of TG2 (2.5 – 20 µg/ml). Control given represents 20 µg/ ml TG2 without Ca^{2+} injection. Data processing involved normalization for well-specific fluorescence and subtraction of control to account for fluorescence bleaching.

TG2-mediated substrate conversion is linear for >30 min and initial reaction rates can be derived from linear regression of first 15-25 data points (Fig. 2A and B).

Conclusion

Reported here are optimized experimental conditions for determination of TG2 isopeptidase activity with the fluorescent model substrate Abz-APE(γcad-Dnp) QEA using the BMG LABTECH microplate readers to produce an assay that is rapid, direct and sensitive. Automated injection of Ca^{2+} for enzyme activation combined with the ability to continuously measure fluorescence intensity over a considerable time period with limited photo-bleaching facilitates the acquisition of kinetic data. Small sample size and plate format make the assay cost-effective and adaptable to high-throughput analysis.

Acknowledgements

This work was supported by grants from Coeliac UK and Arthritis Research UK (18461), and PhD studentships from Cardiff University/the President's Scholarship scheme to MA and AH.

Three assays in one well: antimalarial compound library screening

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- **Metalloaminopeptidases M1, M17 and M18 are attractive new targets for malaria.**
- **Assay developed to identify inhibitors for M1, M17 and M18 in just one well by measuring absorbance and fluorescence over time**

Introduction

Malaria is a tropical disease that is caused by infection of the protozoan parasites of the genus Plasmodium. About 1,000,000 people deaths per year are related to a malaria infection. This explains the very high research interest in finding antimalarial compounds.
Three metalloaminopeptidases are associated with Malaria infection: M1, M17 and M18. All three of them are zinc exopeptidases that catalyze the cleavage of a single amino acid from the N-terminus of a protein. This activity is used by the parasites to digest hemoglobin of the host organism and is absolutely necessary for parasites survival. Compounds that inhibit the peptidases activities can be used to develop a chemotherapeutic strategy.
The nonprofit foundation Medicines for Malaria Venture (MMV) have provided a compound library (MMV400) for the research community. These compounds are known for their antimalarial activity but not necessarily the molecular targets. In this application note we introduce an assay system to identify if any of the MMV400 compounds target one or more of the malarial metallopeptidases M1, M17 and M18.

Assay Principle

To avoid wasting limited amount of compounds, a high-throughput initial screen was developed that is robust enough to narrow down active compounds. As the three enzymes use different substrates it was possible to put the three peptidases, their substrates and one of the 400 compounds into one well.

Fluorescence detection
M1/M17: cleavage of a methylcoumarin substrate results in a fluorescence signal that can be measured at 355 nm excitation and 460 nm emission.

Absorbance detection
M18: cleavage of a nitroanilide substrate results in an absorbance signal at 405 nm.

With the microplate reader from BMG LABTECH, it is possible to accurately monitor absorbance and fluorescence changes over time. All data can be evaluated after the measurements are finished using the MARS data analysis software. One-click assay calculation templates simplify data processing enormously and can be provided custom-tailored by BMG LABTECH.

Materials & Methods

- Multimode reader from BMG LABTECH
- 96-well clear plates from NUNC
- MMV400 antimalarial compound library
- L-Leucine-7-amido-4-methylcoumarin-HCl (Sigma)
- L-Glutamic acid p-nitroanilide (Sigma)

In pretests it was shown that it is possible to use a single buffer for all three assays and that the 3 proteases do not interfere with each other´s activity. The correct concentrations and volumes for the proteases and the substrates were optimized to have a final assay volume of 200 µl including 20 µl of 1 mM compound in 100 % DMSO.

The MMV400 library consists of five 96-well plates containing 400 compounds with nothing in column 1 and 12. The 16 empty wells were used as controls. In 12 control wells all three proteases (PotP) and substrates (PotS) were pipetted but no compounds. In three control wells only the single proteases, M1 or M17 or M18 and the substrate mix (PotS) were present and the remaining control well contained PotP, PotS and a pot of inhibitors (PotI) that were created in our laboratory. The PotP and PotS stock solution were prepared freshly.

Pipetting order
- Add DMSO and PotI to appropriate wells
- Add 80 µl of buffer to all wells with a multichannel pipette
- Leave for 5 min at room temperature
- Add individual proteins (50 µl) to 3 control wells, add PotP (50 µl) to all remaining wells
- Incubate 10 minutes at 37°C with lid on the plate.
- Add PotS (50 µl) to all wells with a multichannel pipette
- Read assay on the microplate reader, pre-warmed at 37°C

Instruments settings

Detection mode:	Fluorescence and Absorbance
Reading mode:	Plate mode kinetic
Number of cycles:	25
Cycle time:	300 sec.

Optic settings

M1/M17 detection:	Ex355/Em460
M18 detection:	405-10

Rev. 03/2013
Keywords: **Compound library, Inhibitor screening, Malaria, Metalloaminopeptidase, Parasites**

Results & Discussion

Fig. 1 shows the change in fluorescence over time related to M1/M17 activity.

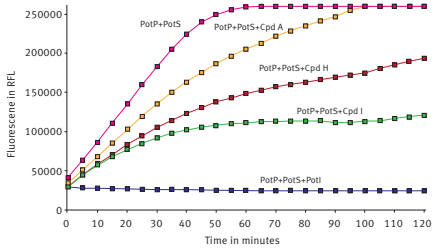

Fig. 1: Kinetic curves obtained from the M1/M17 fluorescent assay. Figure is directly taken from the MARS data analysis software.

Accuracy of the screen is dependent on the PotP+PotS activity alone (without compounds). As expected these controls gave the highest increase of signal over time. The negative control containing inhibitors shows no significant increase of fluorescence over time. Active compounds can be identified by comparing the linear increase/min to the both controls. The lower the fluorescence increase over time the more active is the compound. 25 compounds were identified as having activity against M1 or M17. Some of them are shown in figure 2.

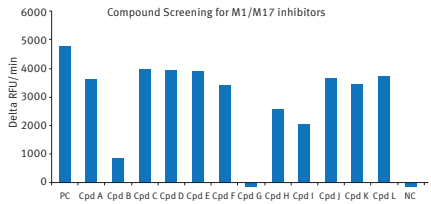

Fig. 2: Screenings results for the fluorescent M1/M17 inhibitor screening. All compound (Cpd) data is compared to the positive control (PC) and the negative control (NC).

No inhibitor for M18 was identified (Fig. 3). This was not surprising as M18 is a highly specific enzyme and 400 compounds is not a big library.

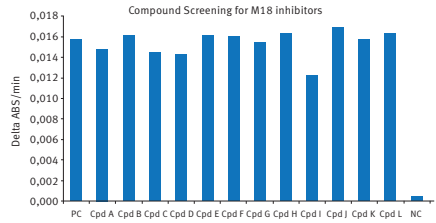

Fig. 3: Screenings results for the M18 inhibitor screening using an absorbance assay. All compound (Cpd) data is compared to the positive control (PC) and the negative control (NC).

A secondary screen was performed to confirm results and identify M1 or M17 as the target. Final results show that only 2 compounds were not inhibitory in the secondary screen. That leads to an 80% success rate for the Pot Assay.

Conclusion

With the help of the BMG LABTECH multimode microplate reader it was possible to develop a robust assay to determine active compounds against the metalloaminopeptidases M1, M17 and M18. By utilizing the instrument to read absorbance and fluorescence over time three assays could be performed in just one well saving limited amount of compounds. With this approach it was possible to screen the whole MMV400 library in just one day with only minimal lab work involved.

A high-throughput, homogeneous, FRET-based assay to detect bacterial membrane-bound enzyme (MraY) activity

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- **Inhibitors of peptidoglycan synthesis represent antibacterial drug targets**
- **A FRET-based assay was developed to find MraY inhibitors**
- **The simultaneous dual emission detection capability of the PHERAstar® *FS* enhances high-throughput**

Introduction

Peptidoglycan is an important structural component of the bacterial cell wall. The continual synthesis of peptidoglycan and remodelling of the cell wall is essential for most bacteria. Since the enzyme phospho-N-acteylmuramoyl-penta-peptide translocase (MraY) catalyzes one of the last cytoplasmic steps in the peptidoglycan biosynthesis, it represents a target for antibacterial drugs. This integral membrane protein catalyzes the attachment of soluble UDP-N-acetyl-muramoylpentapeptide (UNAM-pp) to the lipid undecaprenyl phosphate (C55P) which is membrane bound.

Assay Principle

In this application note we want to present a novel homogeneous FRET-based assay to monitor the activity of MraY. A donor fluorophore (BODIPY-FL) is attached to UNAM-pp (B-UNAM-pp) while the acceptor fluorophore-labelled 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethano-lamine-N-(lissaminerhodamine B sulfonyl) (LRPE), is embedded in micelles which also contain MraY and the lipid substrate C55P (Figure 1).

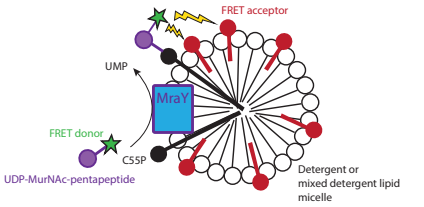

Fig. 1: **Schematic of FRET assay for MraY activity** Micelles containing MraY from E. coli membranes (blue) and C55P (black) are mixed with UNAM-pp (purple) labelled with FRET donor (green). MraY transfers UNAM-pp to C55P bringing the FRET donor into close proximity with the FRET acceptor (red).

MraY translocase activity will attach B-UNAM-pp to C55P bringing FRET acceptor and donor close to one another. Excitation at 485 nm will result in energy transfer, reducing donor fluorescence (at 520 nm) and increasing acceptor fluorescence (590 nm).

Materials & Methods

- PHERAstar *FS* microplate reader (BMG LABTECH)
- Optic module (Ex:485 nm; Em: 520/590)
- Chemicals were obtained from commercial sources
- 384 well, low-volume, black, polystyrene plates (Matrix Tech)

Preparation of UNAM-pp and cloning, expression of MraY in *E.coli* as well as subsequent preparation of membranes from *E.coli* overexpressing MraY is described in the literature.

A 6 µL mixture containing 0.06% Triton X-100, C55P and *E.coli* membranes containing MraY was preincubated for 30 min. Addition of 3 µL of B-UNAM-pp +/- UMP initiated the reaction. Reaction buffer consisted of (final concentrations): 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 50 mM $MgCl_2$, 1 mM dithiothreitol and 0.05% Triton X-100. Final concentration of reactants: 20 mM C55P, 50 µg/mL membrane protein, 2 µM B-UNAM-pp with 0-24 µM LRPE and 0 or 5 mM UMP.

Triplicate MraY assays were performed at room temperature. Fluorescence was excited and simultaneous dual-emission measured using 20 flashes for each reading at a focal height 10.6 mm. Measurements were made every minute for 1 hour and triplicate time courses averaged.

To test the effect of storage at room temperature, MraY assay reagents were stored at room temperature in the dark in the presence or absence of 0.5M trehalose. Reagents were mixed after 0, 2.5 and 5 hours of storage at room temperature and triplicate F_{590}/F_{520} data were collected at the 1 hour time point. Z' calculations could then be obtained from samples with (MAX) and without (MIN) membranes.

To study tunicamycin inhibition, reactions contained 16 µM LRPE, 0 or 15 µg/mL MraY-containing *E. coli* membrane protein, 0.1 µM B-UNAM-pp and 10 µM C55P in assay buffer containing 0.5 M trehalose. Tunicamycin was diluted with assay buffer and average progress curves without membranes were subtracted from the average progresscurves with membranes to obtain $\Delta(F_{590}/F_{520})$ and ΔF_{520} measurements. Percentage inhibition and IC_{50}s were calculated.

Results & Discussion

When the reaction is exposed to fluorescent excitation at 485 nm a time-dependent decrease in 520 and increase in 590 emission is observed (data not shown). Ratiometric measurements were used as they have less noise than individual fluorescence intensity measurements (Figure 2). This is due to the elimination of fluctuations that equally affect both measurements. The ratio change exhibits an increase in a time and LRPE dependent manner.

Rev. 07/2013
Keywords: **Enzyme activity, FRET, Membrane, Peptidoglycan, Simultaneous Dual Emission SDE, Translocase**

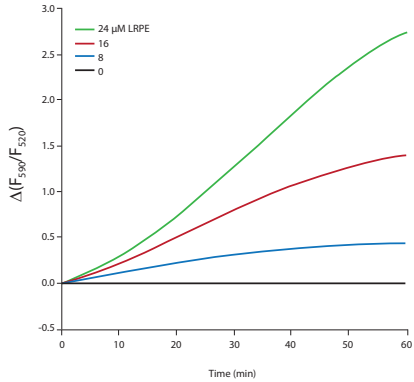

Fig. 2: Ratiometric fluorescence measurement as a function of time and LRPE concentration $\Delta(F_{590}/F_{520})$ is the difference between the 590 nm/520 nm fluorescence emission ratios of reactions without UMP and reactions with UMP.

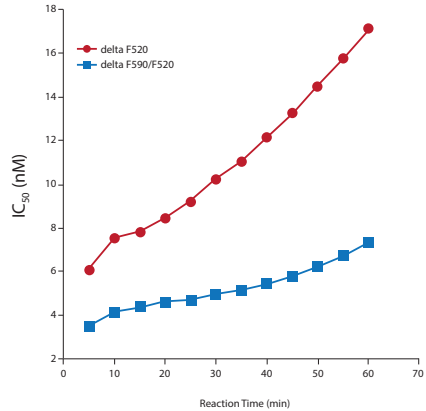

Fig. 4: Inhibition of MraY by Tunicamycin Comparison of IC_{50} versus reaction times measured with Δ(590/520) or Δ(520).

When reactions are performed in presence of 5 mM UMP the change in fluorescence ratio is negligible (data not shown). In the absence of UMP a large increase in fluorescence ratio is observed with time (Fig. 2). This effect indicates that the signal observed in the assay is due to the MraY reaction.

The effect of trehalose, a sugar stabilizer, on storing the reagents at room temperature was also assessed. Figure 3 shows that Z' values decrease when assays are stored at room temperature for as little as 2.5 hours. However the addition of 0.5 M trehalose blocks this effect.

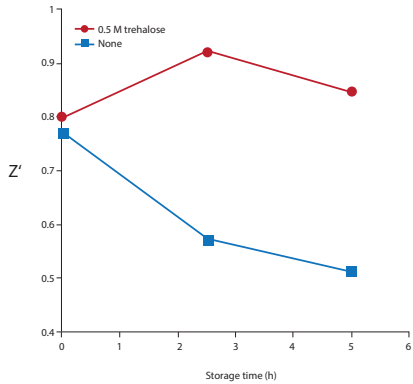

Fig. 3: Z' as a function of reagent storage time and stabilizer Assay reagents were stored at room temperature in the dark for the indicated times. Z' was calculated from data collected after 60 minutes using reactions with (MAX) or without (MIN) membranes.

The sensitivity of this assay to inhibition was studied using tunicamycin (Figure 4). The results show that IC_{50}s measured using $\Delta(F_{590}/F_{520})$ are lower and exhibited less of an increase with reaction time than those measured with ΔF_{520}.

Conclusion

This assay has several advantages over those previously reported. First: it uses no radioisotopes so no special training, handling and disposal are required. Second: it uses a donor fluorophore with an excitation wavelength in the visible rather than UV part of the spectrum. This reduces interference from test compound autofluorescence and absorption. Finally: a FRET assay using the ratio of the emission of acceptor and donor fluorescence intensities upon donor excitation yields greater precision.

The use of $\Delta(F_{590}/F_{520})$ over ΔF_{520} is advantageous due to greater sensitivity to inhibition, resistance to loss of sensitivity to inhibition and a higher signal-to-noise ratio. Furthermore a Z' of greater than 0.7 indicates that this approach will be suitable for high throughput screens.

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Molecular Beacon based helicase assays

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- **Helicase catalysed separation of fluorescently labelled DNA was monitored**
- **The BMG LABTECH microplate reader was used to monitor fluorescence and inject ATP**
- **The MARS data analysis software was used to calculate IC_{50} values**

Introduction

Helicases are motor proteins that bind duplex nucleic acids in order to separate and/or rearrange them. The reaction is fuelled by adenosine tri-phosphate (ATP). Helicases encoded by the genomes of bacteria, viruses, and mammals have diverse functions in processes including DNA replication and repair, RNA transcription, and translation. The importance of helicases in the maintenance of viral and pathogen infection makes helicases attractive drug targets. Small molecule inhibitors of helicases need to be identified. For that purpose the Molecular Beacon Helicase Assay (MBHA) was developed as an improved helicase assay.
This application note describes how to carry out an MBHA to determine the effect of potential inhibitors on the activity of the helicase encoded by the hepatitis C virus (HCV) NS3 helicase (a widely studied model helicase and drug target). A fluorescent double-stranded DNA substrate is used in combination with the BMG LABTECH microplate reader, and the MARS Data Analysis software.

Assay Principle

In the MBHA, the two nucleotide strands of the annealed substrate are molecular beacons that, upon separation, form stem loop structures. One of the nucleic acid strands has both a quencher and a fluorophore at either end so that the two are brought together as the stem loop is formed after strand separation. Thus, a high fluorescence signal is obtained at the beginning of the assay and decreases as the helicase unwinds the substrate. If an inhibiting compound is present, the fluorescence is maintained.

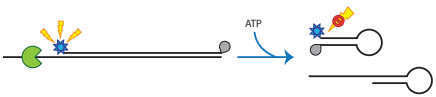

Fig. 1: **MBHA assay principle.** The helicase (green) unwinds the doublestranded DNA substrate in a reaction fuelled by ATP (blue arrows). This causes the fluorescent signal from Cy5 (blue star) to be quenched as the IAbRQ (grey) is brought near Cy5 by the formation of molecular hairpins.

The MBHA can also detect small molecules that interfere in the assay either by absorbing or emitting light at wavelengths similar to Cy5. Interfering compounds either enhance fluorescence or decrease fluorescence relative to negative controls (e.g. DMSO-only). Thus, the MBHA is an ideal tool to determine the inhibitory effect of small molecules on helicase catalysed DNA unwinding.

Materials & Methods

- White, 96-well, half-area microplates (Corning)
- Microplate reader equipped with injectors from BMG LABTECH
- DNA nucleotides with one strand carrying cyanine 5 at the 5' end and IAbRQ quencher at the 3' end were purchased from Integrated DNA Technologies

The expression and purification of the HCV helicase was carried out as previously described.
The DNA strands were annealed to create the Cy5-MBHA substrate. A working stock of 100 nM Cy5-MBHA substrate was prepared in 25 mM MOPS, pH 6.5. Potential helicase inhibitors (compounds B, C and D) were dissolved in DMSO and 8-point, 2-fold dilution series were created.
Reactions contained 25 mM MOPS pH 6.5, 1.25 mM $MgCl_2$, 12.5 nM NS3 helicase, 5 µg/mL BSA, 0.001% (v/v) Tween20, 50 µM DTT, 5 nM Cy5-MBHA substrate, 5% DMSO, various concentrations of compounds, and 1 mM ATP in a final volume of 60 µL. A reaction mix containing all components except inhibitor and ATP was added to the microplate. Inhibitors were added subsequently in a 1:20 ratio to result in a final DMSO concentration of 5%. Quadruplicate negative and positive controls with 5% DMSO and 100 µM primuline (MP Biomedicals) were included. The plate was read using the BMG LABTECH fluorescence plate reader.

Instrument Settings

Measurement Method:	Fluorescence Intensity, top optic
Reading Mode:	Plate Mode Kinetic
Filters:	640-10 / 680-10
No. of cycles:	90-250
Cycle time:	5-20 sec
Injection:	after 10-20 cycles

The gain was set to 85% using a negative control well (DMSO added). The plate was read for approximately 2 minutes before the machine injected ATP at 1:10 ratio (1 mM final, smart dispensing used), then reading continued for approximately a half hour total. Note that cycle time, number of flashes, and number of cycles varies. Efforts should be made to collect many, quality data points. These settings should be adjusted to maintain assay time when different numbers of wells are read. Also note that these buffer conditions are optimized for this particular enzyme. Assay conditions should be optimized for other enzymes.

Rev. 06/2013
Keywords: **Cy5, Helicase, Inhibition dose response, Injectors, Molecular Beacon, Small molecule inhibitors**

Results & Discussion

Several MBHA's were conducted in 96-well using the BMG LABTECH microplate reader to determine the IC_{50} of inhibition of three known HCV helicase inhibitors (compounds B, C, and D).

The potency with which each compound inhibits helicase catalysed DNA unwinding was then evaluated, by first defining a "linear range" in the signal curve (Range 2 in Fig. 2, blue dashed boxes).

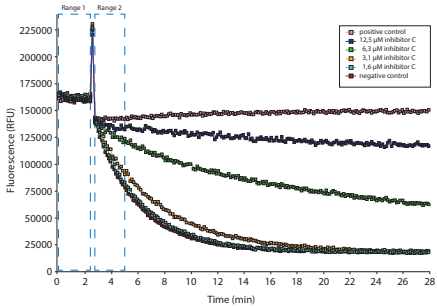

Fig. 2: Raw fluorescence data over time for one inhibitor (compound C) at different concentrations. Note the blue dashed boxes defining data ranges for analysis. Figure was taken directly from the MARS evaluation software.

The kinetic calculation feature of MARS was used to determine the slope of the linear range, which approximates the initial rate of enzyme unwinding. Using the re-calculated concentrations function in MARS, 4-Parameter fits of the slopes to log inhibitor concentration were done to create standard curves (Fig. 3). From these curves the IC_{50} values that represent the compound concentration needed to inhibit 50 % of the helicase activity were calculated.

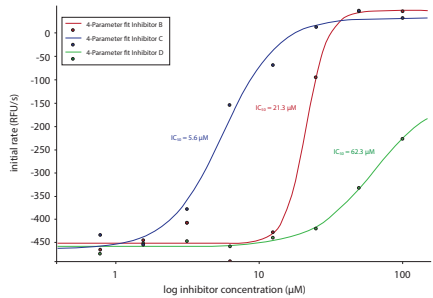

Fig. 3: 4-Parameter fit standard curves based on slope rates and inhibitors concentrations for compounds B, C, and D. The scale of the initial rate is negative because the fluorescence signal is decreasing over time (Fig. 2)

To judge compound interference, average fluorescence of each reaction before ATP addition (Range 1 figure 2) was compared to average fluorescence of reactions performed in the presence of vehicle (DMSO) alone. None of the compounds shown here interfered in the assay. Examples of compounds that interfere with the MBHA, such as the DNA binding compound thiazole orange, have been described elsewhere.

Conclusion

This report demonstrates that the fluorescence mode of the BMG LABTECH microplate reader can be used to follow the unwinding of a double-stranded DNA substrate by helicase enzymes. We also show how the BMG LABTECH MARS data analysis software can be used to calculate the IC_{50} of inhibition.

Following Abeta fibrillization/aggregation in real-time using a FLUOstar® Omega microplate reader

Frank Baumann
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- **Long-term shaking capability of the microplate reader used to monitor Aβ aggregation**
- **Lag times derived from signal curves proved to be a useful measure for the fibrillization process**

Introduction

Aggregation of the amyloid-ß (Aß) peptide is a fundamental hall-mark for Alzheimer's disease. The formation of extracellular senile plaques will lead to synaptic and neuronal damages in clinical demented patients. The aggregation process of Aß peptide is seen as seed driven. These seeds consist of small stable aggegrates of Aß. It is thought that these aggregates are already present in early stages of Alzheimer's even before a patient experiences any symptoms. If this is true, determination of these early aggregates (aggregation seeds) would be an excellent diagnostic tool.
Here we present a cell-free assay (FRANK-Assay = Fibrilization of recombinant Aβ nucleation kinetics) that allows determination of the amount of aggregation seeds from brain tissue homogenates. The assay is run over 2-3 days using the FLUOstar® Omega microplate reader from BMG LABTECH.

Assay Principle

The assay uses Thioflavin T to follow the amyloid formation (Fig. 1). Thioflavin T is a benzothiazole salt that is known to show increased fluorescence when bound to beta sheet-rich structures, such as in amyloid fibrils of Aß.

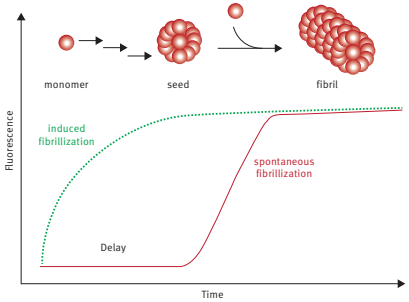


Fig. 1: Fibrillization process followed over time.

Before aggregation or fibrillization can start a critical amount of initial aggregation seeds need to be present or spontaneously formed. This is a thermodynamically unfavoured process even in the presence of excess monomeric Aß, kinetically slow and results in a delay in time before measurable aggregation starts. Once enough seeds have formed a massive and steep increase of fibrillization can be monitored by following the increase of ThT fluorescence due to incorporation into newly formed fibrils.

After some time a plateau is reached indicating the end of the reaction. The delay time is significant and can be shortened by exogenous addition of aggregation seeds. These seeds accelerate increase in fluorescence in relation to their amount.

Materials & Methods

- FLUOstar Omega, BMG LABTECH, Germany
- black, clear bottom 96-well plates, Greiner
- Sealing films, non-sterile, Excel Scientific

All other chemicals were purchased through normal distribution channels.

Donor brain tissue and extract preparation
APP23 transgenic (tg) mice were used as seed donors for all studies. This transgenic mouse model is characterized by an overexpression of mutant human APP. Transgenic APP (amyloid precurser protein) is subject to proteolytic cleavage and gives rise to ß-amyloid peptide which in turn is aggregation prone and results in amyloid plaques consisting of ß-amyloid deposits.

Donor brain tissues were obtained from APP23 tg mice as well as from non tg wild type (WT) mice. After removal the brain was divided into hemispheres. One hemisphere was immediately fresh-frozen (but not fixed) in dry ice while the other hemisphere was immersion-fixed in formaldehyde solution. After finishing the fixation process this hemisphere was frozen on dry ice as well and stored at -80°C until use (Fig. 2).

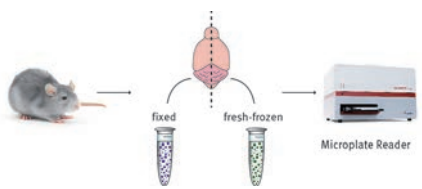


Fig. 2: Method to obtain comparable fixed and fresh-frozen brain samples from either APP23 mice or wild type mice.

Prior to use fixed and fresh-frozen tissues were homogenized, centrifuged and the supernatant aliquoted. For all experiments, a 10 % (w/v) extract was used.

Thioflavin T aggregation assay
1 µl of brain extract, protease inhibitor cocktail (Complete, Roche), 20 µM Thioflavin T, 25 µM Aβ1-40 were incubated in aggregation buffer (50 mM phosphate and 150 mM NaCl) at 37°C. Each brain homogenate was present in

Rev. 12/2014
Keywords: **Alzheimer's, Aggregation, Bottom reading, Fibril formation, Seeding activity, Thioflavin T**

8 replicate wells. The fluorescence increase was measured every 30 min from the bottom of the well. Before each measurement, the microplate was shaken (double-orbital mode) for 30 sec at 500 rpm. The progress of the measurement was followed in the current state window and the reaction was stopped after all samples showed a plateau. Remote control (TeamViewer) was used to monitor and eventually stop the measurement during the weekends. Raw data were fitted and lag times were determined with either using BMG LABTECH's MARS Data Analysis Software or GraphPad Prism.

Instrument Settings

Detection Mode:	Fluorescence Intensity, Plate Mode
Optic:	Bottom optic
Orbital Averaging:	activated, 4 mm diameter
Filters:	440-10/480-10
Number of cycles:	90
Cycle time:	1800 sec
No. of flashes:	10
Temperature:	37°C

Results & Discussion

A typical result for signal curves over time is shown in figure 3:

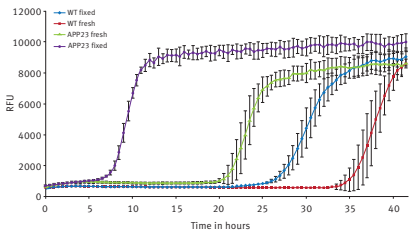


Fig. 3: Signal curves for samples containing either fixed or fresh-frozen wild type or APP23 brain homogenates. Error bars represent deviation of replicate wells within one plate from mean.

All signal curves show a clear increase in fluorescence after a certain time. This increase illustrates the incorporation of Thioflavin T into the newly formed Aβ fibrils. After some time a plateau is reached that is considered as endpoint of amyloid formation and Thioflavin T incorporation process.

The time until the signal starts to increase is the lag time. The MARS data analysis software offers the possibility to create 4-parameter fits of the signal curves from which the lag times are calculated (lag times correspond to the EC20 value of the fit). Initial fibril seeds are formed until the lag time is reached. Considering this, the lag time can be used as a measure to compare different brain homogenates (Fig. 4).

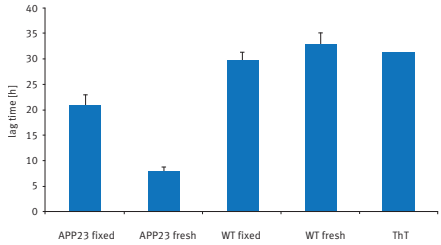


Fig. 4: Lag times created from signal curves shown in figure 3. Fixed and fresh frozen tg and WT mice are compared. As a control thioflavin T only (ThT) was measured on the same microplate (n=1). Error bars refer to 3 biological replicates.

From figure 4 it can be followed that the lag times of the wild type are bigger compared to the lag times obtained for the tg mice. Further a difference can be seen between fixed and fresh-frozen APP23 samples. As expected the fresh samples induce Aβ deposition much faster. Nonetheless, the fixed APP23 samples show compared to the WT a significantly lower lag time indicating that fixation in formaldehyde is not sufficient to prevent Aβ aggregation.

Conclusion

With the help of the FLUOstar Omega microplate reader it is possible to prove that the *in vitro* assay is reliable to detect seeding activity in brain samples. In addition it allows quantitative comparison of seeding activity which with only little effort can be statistically validated. The method is also open for bigger sample cohorts which would easily burst the limited capacity of the *in vivo* approach. Since data can be obtained much easier (no animal surgery and animal permission needed) and earlier (2-3 days vs. 4-6 months) compared to the *in vivo* assay it is meant to replace the latter for qualitative and quantitative seed determination measurements in the future.

Omega Series

Real-time monitoring of genetically encoded redox probes in mammalian cell monolayers

Daria Ezeriña, Bruce Morgan and Tobias P. Dick
Division of Redox Regulation, German Cancer Research Centre (DKFZ), Heidelberg, Germany

- **roGFP2-Orp1 is utilized as an H_2O_2 probe**
- **The BMG LABTECH microplate reader is sensitive enough to permit ratiometric roGFP fluorescence measurements from a monolayer of mammalian cells**

Introduction

Redox processes play an important role in cellular physiology and pathology. A particularly powerful tool for the monitoring of cellular redox changes are genetically-encoded biosensors based on redox sensitive green fluorescent protein (roGFP). RoGFPs contain two cysteine residues engineered to be present on the surface of the protein β-barrel, which are capable of forming a disulphide bond. RoGFP can be made to respond to specific redox species via the genetic fusion of appropriate redox enzymes. For example, fusion of roGFP2 to the thiol peroxidase Orp1 generates an H_2O_2-sensitive probe.

Assay Principle

RoGFP2 exhibits two fluorescent excitation maxima, at 405 nm and 488 nm, when monitoring fluorescence emission at 510 nm. The relative intensities of the two excitation maxima shift in an opposing direction upon reduction or oxidation of the roGFP2 disulphide (Fig. 1). Consequently, by simultaneously monitoring fluorescence emission at the two excitation maxima, it is possible to determine the degree of probe oxidation.

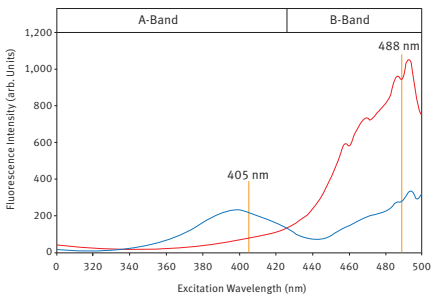


Fig. 1: Redox-dependent changes in the excitation spectrum of roGFP2. Red curve = fully reduced roGFP2. Blue curve = fully oxidised roGFP2.

Fluorescence microplate readers would represent an ideal system for roGFP-based high throughput screening, for example to identify chemical compounds that modulate redox homeostasis. However, microplate reader-based roGFP measurements of cell monolayers require highly sensitive instruments. In this application note we show that the BMG LABTECH microplate reader enables roGFP2-based measurements in mammalian cell monolayers grown in 96-well imaging plates.

Materials & Methods

- BMG LABTECH multimode microplate reader
- Black flat-bottomed 96-well plates (BD Falcon)
- Hydrogen peroxide (H_2O_2) (Sigma, H1009)
- Imaging buffer (130 mM NaCl, 5 mM KCl, 10 mM D-glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM HEPES)

Experimental Procedure

Day 1

Cells stably expressing the cytosolic H_2O_2 probe roGFP2-Orp1 were seeded into a 96-well imaging plate (20,000 cells / well). The same number of non-transduced cells were seeded for use as a background control. The cell number was selected so as to obtain 100% confluence on the day of the measurement.

Day 2

Growth media was removed and the cells were washed twice with PBS, before application of 120 µl of imaging buffer. The response of the probe to an injection of a bolus of H_2O_2 was followed over time.

Instrument settings

Measurement type:	Fluorescence intensity, Bottom reading
Measurement mode:	Plate mode kinetic
No. of cycles:	47
Cycle time:	90 seconds
No. of flashes:	10
Optic settings:	dual chromatic
No. 1:	400 520
No. 2:	485 520
Scan mode:	orbital averaging
Scan diameter:	3 mm
Injection:	using onboard injectors
Injection cycle:	5
Volume:	indiv.
Pump speed:	300 µl/sec

Results & Discussion

With the current state feature of the control software it is possible to follow the reaction progress in real-time. A typical signal curve is shown in Figure 2.

Rev. 05/2014
Keywords: **Biosensor, Bottom reading, H_2O_2, Hydrogen peroxide, Mammalian cells, Redox processes, roGFP**

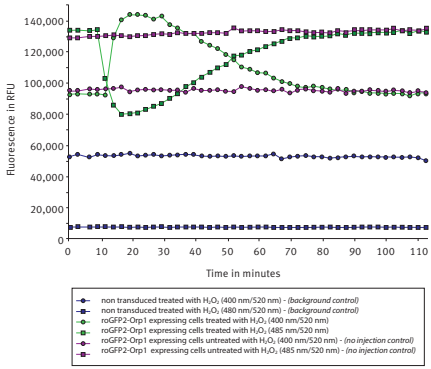


Fig. 2: Signal curves for roGFP2-Orp1 transduced cells before and after injection of hydrogen peroxide in comparison to a non-transfection control and a no injection control.

In the sample expressing the roGFP2-Orp1 construct it can be clearly seen that after H_2O_2 injection the values measured for 400/520 will increase while the values for 485/520 decrease respectively. No effect can be seen in the no construct or no injection control.

The measurement data was processed to obtain degree of probe oxidation values. In figure 3 we monitored the response of the roGFP2-Orp1 probe in a monolayer of confluent lung adenocarcinoma cells, following addition of a bolus of H_2O_2. The sensitivity of the microplate reader makes such measurements easily achievable (Fig. 3).

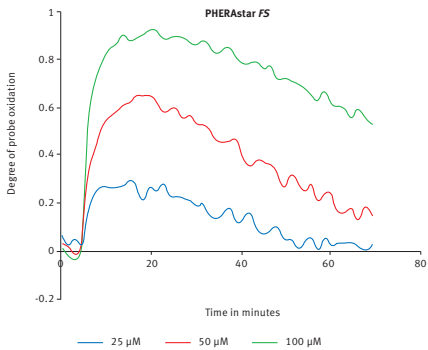


Fig. 3: Lung adenocarcinoma H1975 cells expressing the cytosolic roGFP2- Orp1 probe were grown as a monolayer in 96-well imaging plates. Cells were exposed to H_2O_2 as indicated, and the probe response followed using the PHERAstar® *FS*.

We next assessed the impact of chemical compounds on cellular redox homeostasis. To this end lung adenocarcinoma cells expressing the cytosolic H_2O_2 probe roGFP2-Orp1 were treated overnight with different concentrations of the compound of interest. Subsequently the same cells were challenged with a single bolus of H_2O_2.

As shown in Figure 4, the compound of interest is found to significantly impair cellular recovery from an H_2O_2 challenge in a concentration-dependent manner. This result indicates that the compound disrupts reducing systems inside the cell and thus may be considered a candidate drug to sensitise cancer cells to chemo- or radiotherapy.

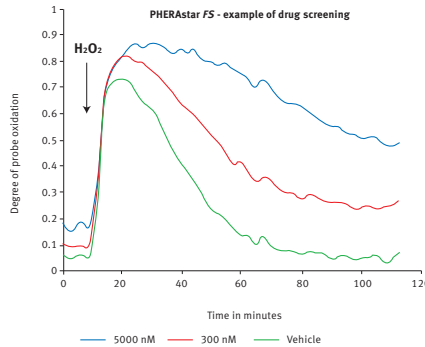


Fig. 4: Lung adenocarcinoma H838 cells expressing the cytosolic H_2O_2 sensor roGFP2-Orp1 were treated overnight with the indicated concentrations of an (unnamed) pharmacological compound. At the indicated time, cells were challenged with a bolus of H_2O_2 (final concentration 75 µM) and the response followed over time.

Conclusion

The microplate reader from BMG LABTECH enables monitoring of the ratio-metric fluorescent response of roGFP2-based redox probes in monolayers of mammalian cells.

Real time calcium flux measurements in iPSC derived 3D heart tissues

Michael Conway and Tetsuro Wakatsuki
InvivoSciences

- **Calcium flux was measured every 0.01 seconds using Fluo-4 and the CLARIOstar®**
- **As a model system for analyzing compound-induced cardiotoxicity, the effects of Cisapride, Thapsigargin, E4031, Nifedipine, are shown**

Introduction

Achieving an approval for a new drug involves extensive testing. While the developers of new drugs not only need to prove that their candidate exhibits a positive effect to treat disease, they also need to confirm that the drug is not dangerous to the overall health of people. Before testing potential drug candidates on humans, a lot of preclinical testing is required to predict potential unwanted side effects that may be caused by taking the drug candidate. Although test results using laboratory animals can predict those side effects, animal models may respond differently to drugs. Researchers are seeking suitable *in vitro* test systems that mimic the physiological state of human tissues and/or organs as close as possible to the *in vivo* response(s).
Traditional test methods involve two-dimensional (2D) cell cultures that can be useful in a first screen. However, a 3D cell culture approach can provide data closer to those obtained in an *in vivo* tissue and minimizes misleading results (false positives and negatives) obtained in 2D cultures.

Engineered heart tissues (EHTs) were fabricated using cardiac myocytes derived from human induced pluripotent stem cells (iPSCs). We show that both human cardiac muscles as well as the EHTs beat at similar rates spontaneously. In this application note we will present how this test model can be used to analyze compound induced changes in calcium handling of cardiac myocytes in 3D tissue, which can indicate potential side-effects of drug candidates.
When an action potential arrives at the cell membrane, the membrane potential (-80mV) gets depolarized. As a result voltage gated L-type calcium (Ca^{2+}) channels open and Ca^{2+} influxes into the cell, which triggers a large release of Ca^{2+} from its internal stores, the Sarcoplasmic Reticulum (SR). This increase in Ca^{2+} initiates a contraction by activating Ca^{2+}-dependent contractile proteins.

The CLARIOstar multimode microplate reader from BMG LABTECH can measure rapid changes (100 data points per second or 1 data point every 0.01 seconds) in fluorescent intensity of the cytoplasmic Ca^{2+} indicator, Fluo-4 (Life Technologies). This was used to quantitatively measure Ca^{2+}-transients.

Materials & Methods

- CLARIOstar, BMG LABTECH
- Fluo-4 (Life Technologies)
- 3D tissue culture micro chamber or plates, MC-8™ or MC-96™ respectively (InvivoSciences)

Hydrogel solution preparation and tissue formation
A general tissue fabrication protocol was published previously. For specific protocols for various tissue types please contact InvivoSciences.

Calcium-indicator measurements with the CLARIOstar
Calcium Transient analysis is performed using commercially available dyes (Fluo-4-AM, Life Technologies). Tissues are bathed in Tyrodes solution containing Fluo-4-AM (15 µM) and Pluronic F127 at 37 °C for 45-60 minutes. Once dye loading is observed by fluorescent microscopy, the loading solution is replaced with just Tyrodes solution and incubated for an additional 20 minutes at 37 °C after which time the samples are ready for study using the CLARIOstar (see instrument settings below). The same approach can be used for tracking 2D Ca-transients optically using our protocol.

CLARIOstar instrument settings

Measurement Method:	Fluorescence Intensity, Well Mode
Monochromator :	Ex: 483-14 and Em: 530-30
Reading Mode:	Bottom Reading
Gain:	1750
Focal height:	2.0 mm
Target temperature:	37°C

Kinetic settings

No. of intervals:	1000
No. of flashes per interval:	1
Interval time:	0.01 sec
Total measurement time per well:	10 sec

Results & Discussion

Periodic changes in fluorescent intensity from the calcium indicator were apparent (Fig. 1A). After smoothing data by filtering using adjacent averaging or Fast Fourier Transfer (FFT) analysis, profiles of Ca-transients from ten (or more) cardiac contractions overlap (Fig. 1B) and their average (Fig. 1C) shows Ca-transients similar to those obtained using trabeculae of human nonfailing myocardium.

Rev. 08/2014
Keywords: **3D cells, Bottom reading, Calcium flux, Cardiotoxicity, Fast kinetic, Heart muscle**

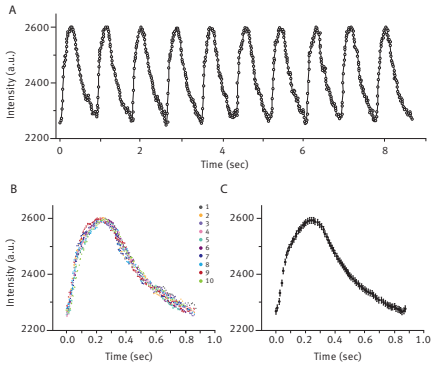


Fig. 1: Calcium (Ca^{2+}) transient measurements using iPSC derived 3D heart tissue and Fluo-4. (A) CLARIOstar microplate reader captures periodic Ca^{2+}-transients that induce cardiac contractions. (B) Overlapping 10 Ca^{2+}-transients of each single cardiac twitch. (C) An average profile of Ca^{2+}-transient (bar=standard error).

The changes in profiles of calcium transients were also apparent when EHTs were treated with various compounds that are known to influence the excitation-contraction coupling (ECC) (Fig. 2) that regulates cardiac contraction through calcium as a second messenger.

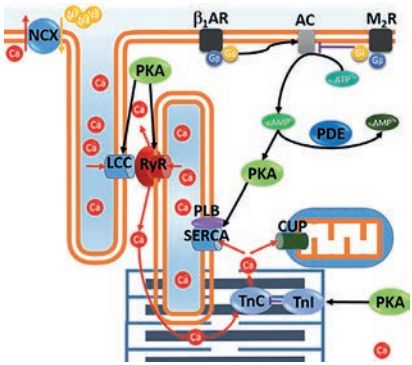


Fig. 2: EC-coupling & regulation. LCC: L-type Ca channel, RyR: Ryanodine receptor NCX: Na-Ca exchanger, PDE: Phosphodiesterase III, PKA: Protein kinase A, TnC/I: Troponin C/I, SERCA: SR Ca pump, PLP: Phospholamban, β1AR: beta adrenergic receptor, AC: Adenylyl cyclase, M2R: M2 Receptor, CUP Ca uniporter.

The results in Figure 3 show similar calcium transient profiles for different concentrations of Cisapride and Thapsigargin, two components that are thought not to affect calcium transients. In contrary, two known effectors (E4031 and Nifedipine) show a clear concentration dependent effect on the shape of the calcium transients.

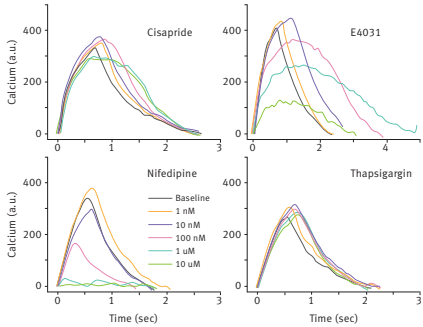


Fig. 3: Calcium Transient Profiles. Human EHTs were treated with increasing concentrations of Cispride, E4031, Nifedipine, and Thapsigargin.

These data suggest that drugs withdrawn from the market changed calcium transients in human EHTs, which may lead to arrhythmia in long-term treatments or in myocardium with significant fibrosis. The analysis of ECC using human EHTs is shown to predict potential safety concerns in drug candidates at the early stage of drug discovery.

Conclusion

The ability to generate EHTs using cardiac myocytes derived from various individuals with different susceptibility to toxicants and drugs could become an important tool to develop safer and more effective personalized treatments.

With the CLARIOstar microplate reader it is possible to measure 100 data points per second. This is very important in these fast kinetic reactions, allowing calcium transient measurements in real time to profile drug-induced changes in excitation-contraction coupling.

Tryptophan quantification using UV fluorescence measurements on the CLARIOstar® multi-mode microplate reader

Mark Gröne and Franka Maurer
BMG LABTECH

- **Tryptophan dilution curve shows linearity over a broad concentration range**
- **Sensitivity was determined to be < 2 nM tryptophan**
- **Combination of filters for excitation and LVF monochromator™ for emission provides excellent UV fluorescence**

Introduction

Tryptophan is one of the 22 amino acids of which proteins are built. It is an essential amino acid in the human diet as it cannot be synthesized by the human organism and has to be externally taken from food. Furthermore, tryptophan is used as a precursor substance for neurohormones, neuro-transmitters as well as for vitamins.

Due to its essential nature, tryptophan is one of the most investigated amino acids. Originally, researchers aimed at identifying nutrients with high tryptophan content for dietary recommendations. Initially, tryptophan was determined by spectrophotometry (Fig. 1) as its aromatic residue enables detection by absorbance (Fig. 2).

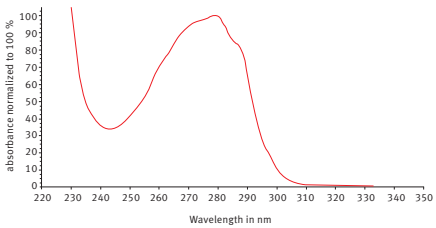


Fig. 1: Tryptophan absorbance spectrum recorded on the CLARIOstar® using 1 nm resolution. In the MARS Data Analysis software the spectral curve was normalized to 100 % for the OD value at 280 nm.

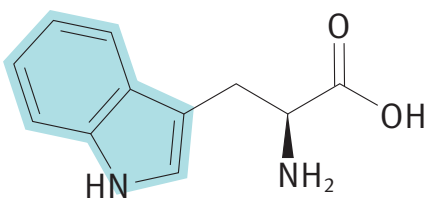


Fig. 2: Structural formula of L-tryptophan. The blue marked part shows the aromatic residue.

With the further development of analytical instrumentation, researchers realized that fluorescence could be exploited for tryptophan determination. Moreover, it was observed that the position of tryptophan within the protein affected its fluorescence. In particular, if the aromatic tryptophan residue is present on the surface of the protein, the fluorescence is much higher compared to the fluorescence that can be captured when tryptophan is located inside the protein. Thanks to this observation, fluorescence could be used to determine the conformational state of a protein and its folding process.

In this application note, we use the CLARIOstar multi-mode microplate reader from BMG LABTECH for the measurement of tryptophan with fluorescence intensity detection. The CLARIOstar can use either filters or combine filters and LVF monochromators™ for an accurate and sensitive detection of tryptophan.

Materials & Methods

- L-tryptophan from SigmaAldrich
- PBS from Biochrom
- 96-well UV-star microplates from Greiner
- CLARIOstar microplate reader from BMG LABTECH

Standard Curve Preparation

A 10 µM tryptophan stock solution was prepared in PBS buffer. From the stock a 10 point dilution was prepared containing the concentrations shown in table 1. Pure PBS buffer served as blank. In every well of the 96-well plate 300 µl of standard or blank was pipetted. Triplicates for every standard and 18 blanks were pipetted.

Table 1: Tryptophan dilution series.

content	Concentration (M)	content	Concentration (M)
S1	5.0E-6	**S6**	5.0E-8
S2	2.5E-6	**S7**	2.5E-8
S3	1.0E-6	**S8**	1.3E-8
S4	5.0E-7	**S9**	6.3E-9
S5	1.0E-7	**S10**	1.3E-9
		Blank	0

Instrument settings

Measurement method:	Fluorescence Intensity, Endpoint mode
Number of fl ashes:	100
Settling time:	0.2

Rev. 07/2014
Keywords: **LOD, LVF Monochromator, Tryptophan, UV fluorescence**

Optical Settings:

Using filter – LVF monochromator combination		
excitation	**dichroic**	**emission**
F: 280-12	automatic monochromator	monochromator 362-25
Using filters		
excitation	**dichroic**	**emission**
F: 280-12	F: Trp-LP	F: 360-20

Automated focus and gain adjustment were performed to obtain the highest dynamic range. The optimal focus for a fill volume of 300 µl resulted in 11.0 mm. Fixed gain and focus values were used for plate-to-plate comparisons with the same filling volume.

Sensitivity (LOD) calculation
The LOD was calculated according to IUPAC standards:

$$LOD = 3^* SD_{blank} / slope_{standard\ curve}$$

Results & Discussion

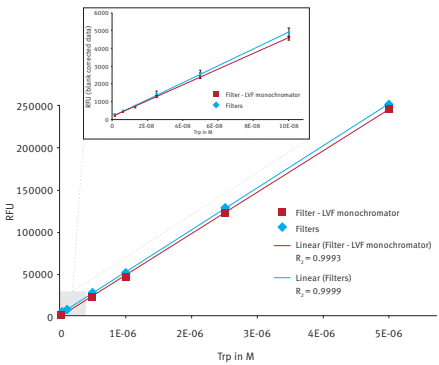


Fig. 3: Tryptophan linearity range measurements using either a combination of filter and LVF monochromator or just filters. The insert zooms into the low nM concentration range.

The standard curve measurement showed a high linearity relation between tryptophan concentration and fluorescence output over a broad concentration range (Fig. 3).
The use either of an emission filter or of the emission LVF monochromator did not affect the sensitivity and accuracy of the measurement, demonstrating that the LVF monochromator has filter-like sensitivity.

Conclusion

The data shown in this application note demonstrate that the CLARIOstar performs accurate and highly sensitive tryptophan fluorescence measurements. The limit of detection was determined to be < 2 nM. This corresponds to a tryptophan concentration of 0.4 ng/ml. This sensitivity can be achieved using either filters for both excitation and emission, or with a filter for excitation and the LVF monochromator for emission.

CLARIOstar®

Looking for a strong promoter for *Physcomitrella patens*

Lucas Schneider[1], Manuel Hiss[1], Stefanie Tintelnot[2] and Stefan A. Rensing[1]
[1]University of Marburg; [2]BMG LABTECH

- **GFP and mCherry used as reporter proteins to research promoter strength in living protoplasts in 96-well format**
- **Using the LVF monochromator, the CLARIOstar® can detect < 100 protoplasts per well**

Introduction

The 35S promoter of the cauliflower mosaic virus is a strong and constitutive promoter that is widely used in plant systems. However, in the moss *Physcomitrella patens*, its promoter strength is weak or all but silent in the dark. Therefore, it is necessary to find promoters that show higher expression levels and that have a more ubiquitous expression over time, tissues and treatments. Of further interest are cell or tissue specific promoters that can be used for targeted gene expression. With expression data available for an increasing number of treatments it is possible to select suitable candidates based on such data.
To test promoter strengths, the protoplasts of the moss *Physcomitrella patens* can be transiently transfected to allow several days of ectopic expression of circular DNA. This moss system can be used to test promoters without the need to produce mutant lines with stable integration. We show that the CLARIOstar can be used to measure the GFP fluorescence within living moss protoplasts and that this assay can be used to compare promoter strengths. To normalize for transformation efficiency a second reporter (mCherry) was introduced into the system.

Assay Principle

The validation of possible new promoters is often done by a promoter-reporter gene construct. A commonly used system fuses the promoter of choice with GFP and then measures the levels of fluorescence after insertion of the construct into the plant. mCherry was selected to be a reporter for transfection efficiency. The GFP/mCherry ratio will be used for further promoter strengths analysis.

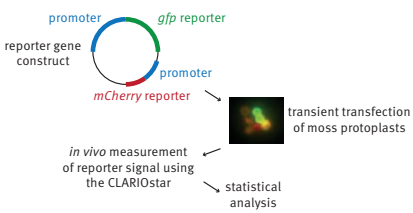


Fig. 1: Standard procedure for validation of new promotors.

Plasmids, carrying promoterA::*GFP* or promoterB::*GFP* or promoterC::*GFP* constructs as first reporter as well as promoter 35S::*mCherry*-constructs as second reporter, are transiently transfected into moss protoplasts. For mCherry-constructs the double 35S promoter was used. The reporter signal is measured after six days of incubation in a CLARIOstar microplate reader. Statistical analyses of the GFP/mCherry-ratio allow the determination of the promoter strength.

Materials & Methods

- CLARIOstar microplate reader from BMG LABTECH
- Greiner CELLSTAR® medium binding 96 well half area polystyrene plates, black with transparent flat bottom
- Stable and transiently GFP and mCherry transfected protoplasts of *Physcomitrella patens* in regeneration medium
- Regeneration medium contains the following substances: 250 mg/L KH_2PO_4, 250 mg/L KCl, 250 mg/L $MgSO_4$ x 7 H_2O, 1000 mg/L $Ca(NO_3)_2$ x 4 H_2O, 12.05 mg/L $FeSO_4$ x 7 H_2O, 50 g/L glucose and 44 g/L mannitol)

Fluorescence intensity measurements were performed with transfected *Physcomitrella patens* protoplasts. Sample volumes of 100 µL (up to 30,000 protoplasts) were placed into black 96 well microplates with transparent bottom. After wavelength optimization the monochromator of the CLARIOstar was set up to measure GFP and mCherry fluorescence using one test protocol (ex/em for GFP: 480-12/519-15 nm; for mCherry: 562-12/603-20 nm). The samples were detected using the bottom optic, orbital averaging with 2 mm diameter and 15 flashes per well. Regeneration medium was used as the blank. All promoter measurements were background subtracted. The ratio of GFP and mCherry signals was calculated to normalize the data for the transfection efficiency.

Well Scanning
With the well scanning function of the CLARIOstar it was possible to check the distribution of protoplasts in the well. The instrument was set up to scan from the bottom using a scan matrix of 20 x 20 data points.

Results & Discussion

The well scan figure shows that the protoplasts are not evenly distributed in the well (Fig. 2).

Rev. 11/2014
Keywords: **GFP, mCherry, Moss, Reporter gene, Transient transfection, Well scanning**

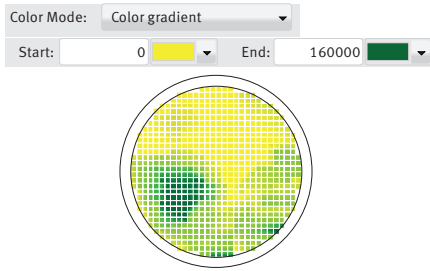


Fig. 2: Well scanning figure of protoplasts that express GFP. Measured at 480/519 nm.

Because of this finding we have assumed that it is more useful measuring data points in a small circle and average them afterwards instead of only measure in the middle of the well. The orbital averaging function in the BMG LABTECH control software allows for such a measurement, a diameter of 2 mm was found to give the most stable results.

After optimal wavelength settings were determined a protoplast dilution of single expressing cell lines was carried out in order to find the optimal number of protoplasts per well. Figure 3 shows standard curves for GFP and mCherry expressing cells.

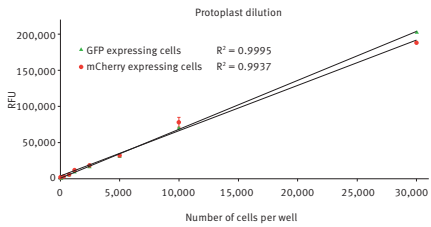


Fig. 3: GFP protoplast expression measured at 480/519 nm. mCherry protoplast expression measured at 562/603 nm.

Both protoplasts dilution curves show a good linearity over a broad number of cells per well range.

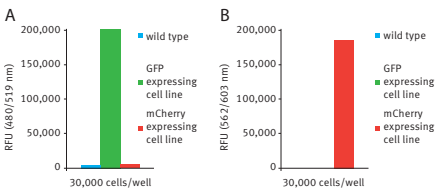


Fig. 4: A GFP fluorescence measured for wild type, GFP and mCherry expressing cell lines. B mCherry fluorescence measured for wild type, GFP and mCherry expressing cell lines.

Figure 4 and B compare the fluorescence intensity values obtained for wild type, GFP and mCherry expressing protoplast samples. In the wavelength range of GFP (480/519 nm) there is a certain amount of background fluorescence present in both, the wild type and the mCherry expressing cell line (Fig. 4A). In the wavelength range of mCherry (562/603 nm) there is nearly no background signal in wild type and GFP expressing cell line (Fig. 4B). It was possible to detect a significant signal in as low as 150 GFP expressing protoplasts and resp. < 100 mCherry expressing protoplasts per well.

Promoter strength analysis

For promoter strength analysis 4,000 protoplasts per well were used that stably expressed both GFP and mCherry. Figure 5 shows results for different promoter:: GFP-constructs.

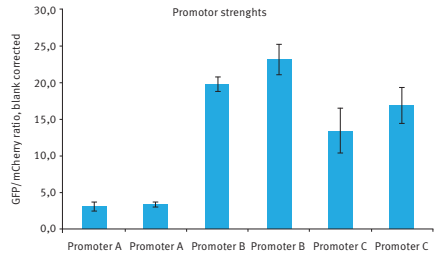


Fig. 5: GFP/mCherry ratio of transiently transfected protoplasts. Error bars show the standard deviation of technical replicates.

Transfection experiments using different promoters were evaluated with the help of the GFP/mCherry ratio. From Figure 5 it can be followed that the Promotor B is the strongest promoter tested.

Conclusion

We introduce a transient assay using BMG LABTECH's CLARIOstar that can be used to measure GFP and mCherry fluorescence in living protoplasts in a microplate format. With such an assay, it is possible to perform expression studies in transiently transfected cells. It is a really fast and simple method compared to the time-consuming process of post transfection selection using antibiotics. In the future, this assay format could be used as a standard method for screening transiently transfected protoplasts.

Quant-iT™ PicoGreen® dsDNA assay for nucleic acid quantification

Silke Angersbach, Ron Earp, Mark Gröne and Franka Maurer
BMG LABTECH

- **Double stranded DNA quantification performed in down to 2 µl using the LVis Plate**
- **High linearity and sensitivity over a broad DNA range**
- **Easy to use UV absorbance alternative assay that can be miniaturized and adapted to high-throughput systems**

Introduction

The quantification of nucleic acids samples is a major need in any genetic and molecular biology laboratory. Next to well-known UV absorbance assays (e.g. 260/280 nm), a lot of different fluorescence intensity based DNA quantification assays are offered. These assays are not only more sensitive than absorbance assays, they are also able to differentiate between double-stranded and single-stranded DNA, as well as RNA.
Aside from sensitivity and specificity, the demand for very low volume measurements is emerging. This is challenging for assays as well as for the detection instrumentation. In this application note we present DNA quantification data obtained on the low volume LVis Plate, as well as 96- and 384-well microplates.

Materials & Methods

- Quant-iT™ PicoGreen® dsDNA assay, LifeTechnologies
- Low volume LVis Plate, BMG LABTECH
- Black 96-well and 384-well microplates, Greiner

Sample preparation

All necessary reagents such as detection reagent, TE buffer, DNA standard (Lambda DNA standard) are provided with the kit. From a 20x TE buffer stock solution a 1x TE buffer was prepared using HPLC grade water. The PicoGreen® reagent was diluted 1:200 by using 1x TE buffer. dsDNA standards were diluted with 1x TE buffer to obtain a concentration range of 2 to 1000 ng/ml. DNA standards were pipetted in 6 replicates, adding the same amount of PicoGreen® reagent into each well leading to the final volumes: 200 µl in 96-well plates, 20 µl in 384-well plates and 2 µl in the LVis Plate. The blank consisted of TE buffer.

Instrument settings

	FLUOstar®/ POLARstar® Omega	CLARIOstar®	PHERAstar® *FS*
Filter settings	Ex485 Em520	Ex485 Em520	Optic Module: FI 485 520
Monochromator settings		Ex: 483-14 Em: 530-30	

All other settings are default settings for a fluorescence endpoint test protocol.

Results & Discussion

Fluorescence scan of PicoGreen® reagent bound to DNA

In order to determine useful monochromator settings for the CLARIOstar, an excitation and emission scan was done for the DNA bound PicoGreen® reagent. The spectra scan result is shown in Fig. 1.

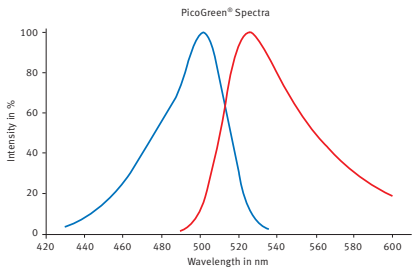


Fig. 1: CLARIOstar spectral scan of PicoGreen® reagent bound to DNA. The excitation scan (blue line) was recorded between 400 and 540 nm with a fixed emission wavelength at 550 nm. The emission was scanned between 490 and 600 nm (red line) while a fixed excitation wavelength of 460 nm was used.

The scan showed an excitation maximum at 502 nm and an emission maximum at 524 nm. For further measurements, the highest signal/blank ratio was determined by varying the band widths for excitation and emission.

96-well

In 96-well plates the standard curves obtained using either filters or the monochromator are very similar (Fig. 2). With filters the slope of the standard curve is slightly higher.

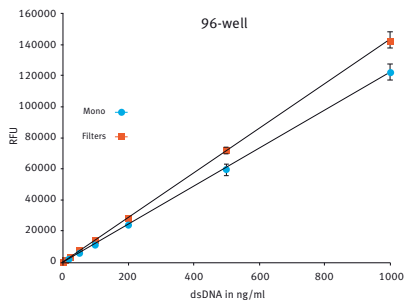


Fig. 2: PicoGreen® assay comparison using either filters (red) or the LVF Monochromator™ (blue) in 96-well format in the CLARIOstar. The final volume in the well was 200 µl.

Rev. 12/2014
Keywords: **DNA quantification, Low-volume, LVis Plate, PicoGreen, Quant-iT**

384-well

To miniaturize the assay, the well volume was decreased to 20 µl. The resulting standard curve in 384-well small volume plate (Fig. 3) was comparable to the data obtained for 96-well plates.

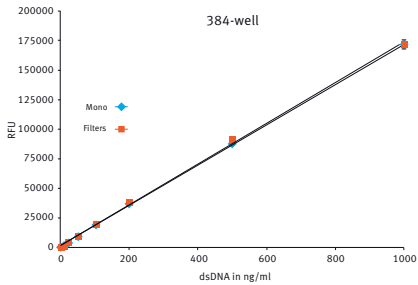


Fig. 3: PicoGreen® assay comparison using either filters (red) or the LVF Monochromator™ (blue) in 384-well format in the CLARIOstar. The final volume in the well was 20 µl.

Very good linearity was achieved for the CLARIOstar® using either filters (R^2 = 0.9999) or LVF Monochromators™ (R^2 = 0.9998).

LVis Plate results

With the LVis Plate it is possible to miniaturize the assay further. This special plate was developed to use a volume of as little as 2 µl (Fig. 4).

Fig. 4: LVis Plate from BMG LABTECH.

Originally developed to do DNA and protein UV absorbance quantification the LVis Plate, used in the CLARIOstar or PHERAstar® *FS*, is an excellent tool to determine the DNA content of samples in fluorescence intensity mode. Results for the PicoGreen® assay in the LVis Plate are shown in Fig. 5.

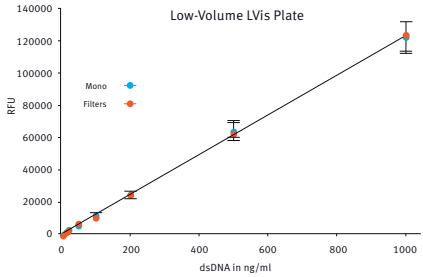


Fig. 5: PicoGreen® assay comparison using either filters or the LVF Monochromator™ in the LVis Plate. The final volume in the well was 2 µl.

Conclusion

The standard curve data show that the PicoGreen® assay can be measured with high linearity and sensitivity over a broad DNA concentration range. For the CLARIOstar, the LVF Monochromator™ shows filter-like performance. For example, the sensitivity in 384- well plates was calculated to be about 3 pg/well for both filters and LVF Monochromators™.

With the help of the PicoGreen® assay, any preferred volume, between 2 µl in the LVis Plate or standard volumes in 96- and 384-well plates, can be used to determine the DNA concentration of samples.

PHERAstar® *FSX*
PHERAstar® *FS*

CLARIOstar®

Omega Series

High-speed FRET-based SNP genotyping measurement on the PHERAstar®

Phil Robinson[1] and Franka Ganske[2]
[1]KBiosciences (now LGC) [2]BMG LABTECH

- **FRET based SNP genotyping using the KASP™ system**
- **Optimized optical modules with dual emission**
- **Improvements in speed and accuracy of measurement**

Introduction

Single Nucleotide Polymorphisms (SNP`s) have become an invaluable tool in the field of Genetic Research. They are employed in a wide range of scientific fields from Pharmacogenetics to animal breed identification through to disease gene mapping. They are a popular choice of genetic marker due to their ease of assay and analysis, however many standard assays remain relatively expensive to use in high throughput. In this application note we show the use of the PHERAstar® multimode plate reader for measurements in both SNP Genotyping chemistry development and high throughput production genotyping. The KASP™ system, developed by KBiosciences (now LGC), was used to assess the performance enhancements of the PHERAstar, using a new SNP genotyping specific optical module with dual emission created by BMG LABTECH (Figure 1). Using two photomultipliers allows for improved accuracy and reduced read times by 30%.

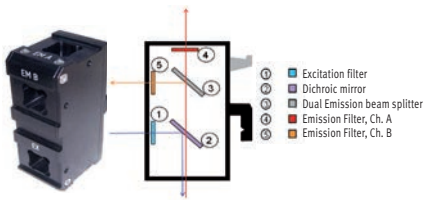


Fig. 1: SNP Genotyping optical module with dual emission wavelength detection (SDE module).

Materials & Methods

All materials were sourced from Sigma Aldrich, with the exception of 384- and 1536-well microplates which were produced in house (Part Numbers – Pro 384 and 1536 respectively). Oligonucleotides were also sourced from Operon, Germany.

The chemistry used was a KBiosciences proprietary system based on a homogeneous Fluorescence Resonance Energy Transfer (FRET) detection system, using allele specific PCR. Briefly, two oligos are designed specific to each allele of the SNP. Each one of these oligos is tailed with an 18bp sequence distinct from each other. Also included in the reaction is Taq polymerase, dNTPs, an internal standard dye (Rhodamine X, (Rox)) and reverse primers.

Modified versions of Taq polymerase are unable to extend primers that are mismatched at their 3´terminal base. This is used to discriminate the two alleles. The reaction is monitored by the creation of fluorescence from two novel FRET reporter oligos that are included in the reaction. All reactions were conducted in a volume of 1 µl (1536-well plates) or 2 µl (384-well plates) and thermal cycled in a Hydrocycler thermal cycler (KBiosciences, Basildon, UK).The liquid handling was performed using a Deerac Equator™ GX8 (Deerac Fluidics, Dublin, Ireland).

Results & Discussion

SNP based genotyping is essentially a qualitative technique with the output being a cluster plot. In the case of the PHERAstar assessment an initial evaluation was carried out to determine the optimal conditions for reading. During this phase the effect of gain, excitation and emission filter choice and number of flashes per well were determined. Dual emission modules offer the great advantage to read the internal standard (ROX) and the allele specific dyes (FAM and VIC) together. Optimal modules were determined to be:

FAM/ROX	Excitation 485 nm	EmissionA 610 nm	EmissionB 520 nm
VIC/ROX	Excitation 520 nm	EmissionA 610 nm	EmissionB 560 nm

Figure 2 and 3 show the results obtained from measurements with different emission modules. The results show, sharper clusters are obtained when the dual emission module is used.

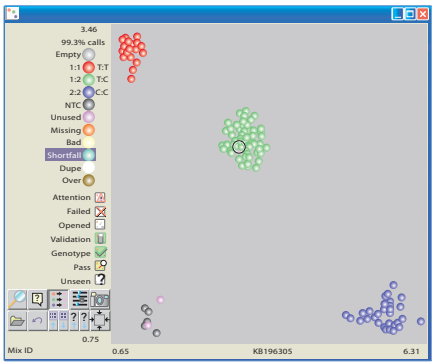


Fig. 2: Cluster plot from the KBiosciences Genotyping LIMS package from a measurement using the single emission module.

Rev. 12/2014
Keywords: **Allele specific PCR, KASP, Polymorphism, Simultaneous Dual Emission SDE, SNP genotyping, Taq polymerase**

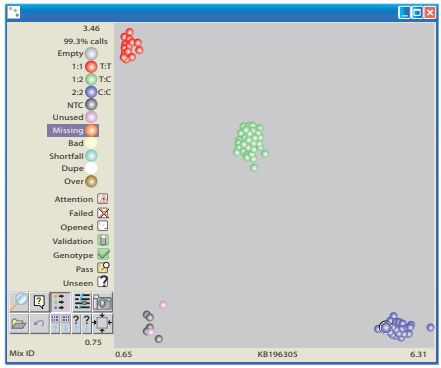


Fig. 3: Cluster plot from the KBiosciences Genotyping LIMS package from a measurement using the dual emission module.

Conclusion

The PHERAstar has always performed this application well in the past but by introducing the SDE modules a further significant improvement in performance was achieved. In addition to this, the implementation of the dual optical modules at the KBiosciences production facility has resulted in an increased throughput of 30% due to the having to read each well only twice (Fam/Rox, then Vic/Rox) instead of each Fluor separately.
Finally we have noticed that since implementation of the dual modules the mis-read rate of the PHERAstar has decreased to a level that is no longer measurable. After the initial evaluation, the whole production site of PHERAstars at the KBioscience facility has been upgraded to dual modules.

Background

LGC's Genomics division combines with KBioscience
LGC, the international science-based company and market leader in analytical, forensic and diagnostic services and reference standards, announces that LGC's Genomics division and KBioscience have come together to create a market-leading genomic services and solutions provider.

About KBioscience
KBioscience was a UK-based technology company focused on SNP Genotyping chemistry and novel instrumentation. Set up in 2002 to exploit the use of laser welding as a service-based science company in the SNP genotyping market, KBioscience has developed its own range of SNP genotyping chemistry and novel instrumentation.

PHERAstar® *FSX*
PHERAstar® *FS*

ORAC assay to determine antioxidant capacity

Franka Ganske
BMG LABTECH

- **Antioxidants are able to neutralize Reactive Oxygen Species (ROS)**
- **ORAC assay uses Trolox® as reference substance**
- **MARS Data analysis software with predefined template for quick ORAC evaluation**

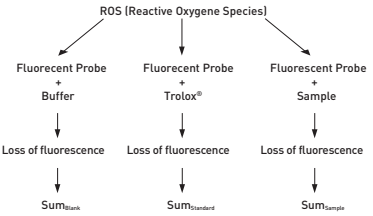
Introduction

In all oxygen consuming cells, metabolism and oxidative stress generate several intermediates and byproducts that are collectively known as reactive oxygen species (ROS). ROS are necessary intermediates in the human body, but they are also involved in the aging process and in the development of many degenerative diseases, including cancer, heart disease, Alzheimer's and Parkinson's. ROS are dangerous to cellular structures and functional molecules (i.e. DNA, proteins, lipids) as they act as strong oxidizing agents or free radicals. Biological antioxidants are able to dispose of ROS; however, they are not completely effective in eliminating all of the free radicals, oxygen ions and peroxides that can do damage to the body. Furthermore, ROS can be generated from exposure to other external sources such as cigarette smoke, pollutants, chemicals and environmental toxins.

One standardized method for determining the antioxidant capacity of a substance is the ORAC (oxygen radical absorbance capacity) assay. The ORAC assay is based upon the inhibition of the peroxylradical-induced oxidation initiated by thermal decomposition of azocompounds such as [2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH)]. In this manner, the ORAC assay uses a biological relevant radical source and it combines both inhibition time and degree of inhibition into one quantity. Recent modifications to this assay include the use of fluorescein as the probe, the adaptation to a high-throughput format, and the ability to measure the lipophilic, hydrophilic, and total antioxidant capacity of a substance. These modifications, along with no washing steps, have greatly simplified the ORAC assay; thereby making it ideally suited to measure the antioxidant capacity of a substance.
Herein we describe the application of the ORAC-FL assay using Trolox® (a water-soluble analogue of vitamin E) as a standard by which all other antioxidant compounds are compared.

Assay Principle

Over time ROS, generated from the thermal decomposition of AAPH, will quench the signal from the fluorescent probe fluorescein. The subsequent addition of an antioxidant produces a more stable fluorescence signal, with signal stability depending on the antioxidant's capacity (Fig. 1). The data points are summarized over the time by the evaluation software. This is then compared to the standard, Trolox®, and is expressed as micromoles of Trolox® equivalents (TE) per gram or per milliliter of sample (µmole of TE/g or µmole of TE/mL).



Antioxidant Capacity relating to Trolox® = $(Sum_{Sample} - Sum_{Blank}) / (Sum_{Standard} - Sum_{Blank})$

Fig. 1: ORAC Assay Principle.

Materials & Methods

All materials were obtained through normal distribution channels from the manufacturer stated.

- Costar® 96 well black opaque plate, Corning Costar
- Fluorescein Sodium, 6-Hydroxy-2,5,7,8-tetra-methylchroman-2- carboxylic acid (Trolox®), L (+)-ascorbic acid, Epicatechin gallate, [2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH)] were obtained from Sigma-Aldrich

Test Protocol
Different dilutions of Trolox® (200 µM – 12.5 µM) and sample compounds (ascorbic acid and epicatechin gallate, two known antioxidants) were prepared in phosphate buffer (10 mM, pH 7.4). All solutions were and should be prepared fresh daily.
In every working well the following was pipetted in triplicate:

- Fluorescein, 150 µl of a 10 nM solution
- For standard, 25 µl of Trolox® dilution
- For sample, 25 µl of sample dilution
- For blank, 25 µl of phosphate buffer

The microplates were sealed followed by incubation for 30 min at 37°C in a Thermostar microplate incubator without shaking. Alternatively, the BMG LABTECH microplate reader itself can perform the incubation step.
After incubation was finished, fluorescence measurements (Ex. 485 nm, Em. 520 nm) were taken every 90 sec to determine the background signal. After 3 cycles, 25 µl (240 mM) of AAPH was injected with the help of onboard injectors. Alternatively AAPH can also be added manually with a multi-channel-pipette.

Rev. 12/2014
Keywords: **Antioxidant, Food, Injectors, ORAC, ROS, Trolox**

This has to be done as quickly as possible since the ROS-generator displays immediate activity after addition. The test was resumed and fluorescent measurements were taken up to 120 minutes.

Instrument settings

	FLUOstar®/ POLARstar® Omega	CLARIOstar®	PHERAstar® *FS*
Detection mode	Fluorescence Intensity		
Method	Plate mode kinetic, top optic		
Optic settings	Ex-Filter: Ex485 Em-Filter: Em520	Ex-Filter: Ex485 Em-Filter: Em520 or Monochro-mator: Ex: 483-14 Em: 530-30	Optic module: FI 485 520
Cycle Time	90 seconds		
Number of cycles	80		

Results & Discussion

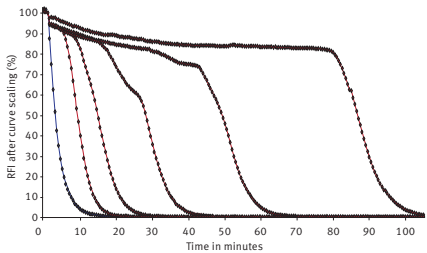


Fig. 2: Signal curves for different Trolox® concentrations (red graphs) and a blank without Trolox (blue graph) recorded on the PHERAstar *FS* in 384-well format. The curves were normalized to 100 %. The 100 % value is the maximum value that is obtained directly after injection of AAPH.

Figure 2 shows Trolox® signal curves (relative fluorescent units versus time) at different concentrations. After 3 cycles AAPH was added, which lead to a loss in fluorescence signal that depended upon the concentration of Trolox®.
Since the sample concentrations are known, the software allows the user to simultaneously look at calibration curves. Figure 3 depicts the blank-corrected linear regression curves of Trolox®, ascorbic acid and epicatechin gallate. Graphically one can see that ascorbic acid is a weaker antioxidant than Trolox®, whereas epicatechin gallate is a much stronger one.

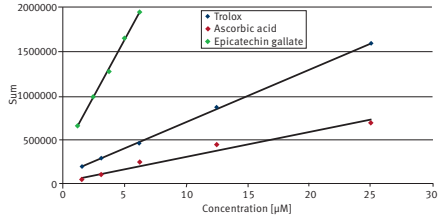


Fig. 3: Blank-corrected linear regression curves of Trolox®, ascorbic acid and epicatechin gallate. The data points were summed over time and were plotted on the y-axis vs. concentration.

To obtain the values for Trolox® equivalents (TE) of antioxidants with known concentration over the desired concentration range one can divide the slopes of the regression curves:

$$\text{TE over considered concentration range} = \frac{\text{slope regression curve (sample)}}{\text{slope regression curve (Trolox®)}}$$

In the case of compounds with unknown concentrations, the software calculates the Trolox® equivalents of a special dilution using the Trolox® calibration curve.

Conclusion

The ORAC assay is a common and popular tool used to determine the antioxidant capacity of any substance. With the help of the easy-to-use MARS Data analysis software, the antioxidant capacity of a substance can be directly estimated by comparison to the standard curve of Trolox®. The progress of each reaction can be followed in realtime using the current state option. Furthermore, the use of onboard injectors allow for consistent and reproducible data.

Transcreener® ADP² FI assay performed on BMG LABTECH microplate readers

Franka Ganske[1] and Meera Kumar[2]
[1]BMG LABTECH [2]BellBrook Labs

- **Transcreener® ADP² FI assay kit is a simple one-step competitive red fluorescence immunoassay based on the detection of ADP**
- **BMG LABTECH microplate readers are compatible with this assay**

Introduction

The Transcreener® technology was developed by BellBrook Labs to quantify the production of ADP during enzyme reactions. Different detection modes are possible in combination with the Transcreener® method (* FI, FP and TR-FRET). This application note focuses on the homogeneous, competitive red ADP² fluorescent intensity (FI) assay.
The assay is based on the detection of ADP, therefore is compatible with any enzyme class that produces ADP, including protein, lipid, and carbohydrate kinases, ATPases, DNA helicases, carboxylases and glutamine synthetases. The assay is a simple one step homogeneous detection assay that can be applied to a wide range of ATP concentrations (0.1 to 100 µM ATP).
In this application note we will show that combination of the Transcreener® chemistry with the BMG LABTECH microplate readers provide excellent Z'values, indicating a robust assay and instrumentation.

Assay Principle

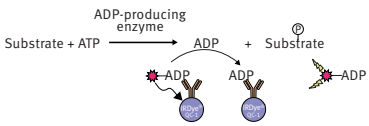


Fig. 1: Transcreener® ADP² FI Assay Principle.

After the enzymatic reaction is finished, an ADP Alexa594 tracer bound to the ADP2 monoclonal antibody, which is conjugated to an IRDye® QC-1 quencher (licensed from LI-COR®), is added. Accumulated ADP from the reaction will eventually displace the ADP-tracer from the antibody-quencher complex into the solution. Here the ADP-tracer complex becomes un-quenched resulting in an increase in fluorescence intensity. The ADP created during the enzyme reaction is proportional to the fluorescence signal.

Materials & Methods

- Black 384-well small volume plates from Greiner bio-one, Germany
- Transcreener ADP² FI Assay Kit for 96-wells or 384-wells from BellBrook Labs

To show the potential of the instrumentation, ADP/ATP standard curves were created to mimic an enzyme reaction. For that 10 µM ADP and 10 µM ATP stock solutions were combined to give 15 standards with an ADP range from 0 to 10 µM.
For 384-well plates the reaction mix consisted of 10 µL of ADP/ATP dilution and 10 µL of ADP detection mixture. The final concentration of tracer in the well was 4 nM. The final concentration of antibody conjugated to the QC-1 quencher depends on the ATP concentration. For the 10 µM ADP/ATP dilutions a final antibody concentration of 5 µg/mL per well was used as recommended in the Transcreener® manual.
As controls, a high RFU control and a low RFU control were prepared:

High RFU control = Positive control
4 nM tracer in 0.5x buffer

Low RFU control = Negative control
Detection mix, 4 nM tracer and 5 µg/mL antibody conjugated to the QC-1 quencher

After the addition of the detection mixture to the standards a one hour incubation at room temperature follows. The plate was then inserted into a plate reader, the gain was adjusted to 10% of the positive control and fluorescence was measured.

Instrument settings

	POLARstar® Omega	CLARIOstar®	PHERAstar® *FS*
Detection mode	Fluorescence Intensity		
Method	Endpoint, Top optic		
Optic settings	Excitation Filter: 580-10 Emission Filter: 620-10	Monochromator settings 575-20/630-40	Transcreener specific FI optic module: FI 580 620

Data Analysis
The Z'-value, a standard for evaluating HTS methods, is calculated using the formula:

$$Z' = 1 - \frac{3\sigma_p + 3\sigma_n}{|\mu_p - \mu_n|}$$

where μ_p = mean of "positive control" (max ratio), μ_n = mean of "negative control" (min ratio), and σ = the corresponding standard deviations.

Rev. 12/2014
Keywords: **ADP², Alexa594, ATP, Transcreener, Z' value**

Results & Discussion

Figure 2 and 3 show a 15 point ADP/ATP standard curve monitored either on the PHERAstar FS or Omega plate reader in 384-well format.

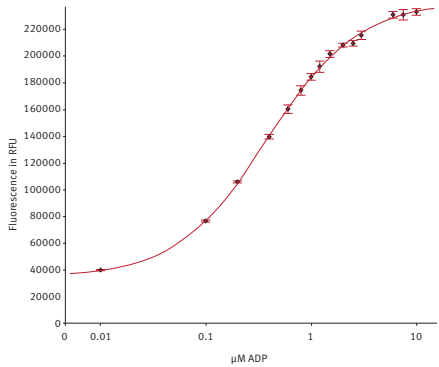


Fig. 2: 10 µM ADP standard curve measured in 5 replicates using a PHERAstar *FS* in 384 well format (20 µL). The concentration of 0 µM ADP was set to 0.01 µM to allow logarithmic scaling.

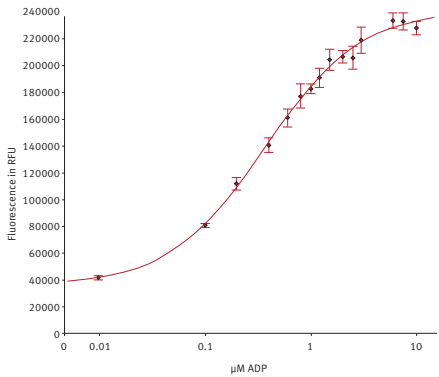


Fig. 3: 10 µM ADP standard curve measured in 5 replicates using a POLARstar Omega in 384 well format (20 µL). The concentration of 0 µM ADP was set to 0.01 µM to allow logarithmic scaling.

The standard curves for PHERAstar *FS* and CLARIOstar look very similar (data for CLARIOstar not shown). For the Omega the error bars are a slightly higher in the standard curve. This is due to the different optical system used in Omega instruments.

With the MARS Data Analysis Software that comes with every BMG LABTECH reader it is possible to calculate different assay parameters, like Z´values with just one mouse-click. Table 1 summarizes Z´value results for the different instrumentation.

Table 1: Z´ values for the Transcreener assay compared for different BMG LABTECH instrumentation.

	PHERAstar *FS*	CLARIOstar	POLARstar/ FLUOstar Omega
Z´value (384-well) at 10 % conversion using 5 flashes	0.89	0.83	0.80

Monochromator-based instruments like the CLARIOstar are often thought to have a significant higher read time to perform assays compared to filter-based microplate readers. The read time is depending on the number of flashes. In Table 2 this relation is shown as well as the corresponding Z' values.

Table 2: CLARIOstar® assay performance at 10 % conversion 10 µM ATP.

Flashes	1	5	10	20	50
Read Times (min) whole 384 well plate	1:23	1:31	1:43	2:02	3:01
Z'Factor at 10 % ATP conversion	0.67	0.83	0.85	0.87	0.89

The criteria to get the Transcreener® FI certification is to achieve a Z' factor of 0.7 at 10 % ATP conversion. As shown in Table 2 this Z' value is already reached if only 5 flashes are used resulting in a read time of 1:31 for a whole 384-well plate. This indicates that measurements with the CLARIOstar using the LVF monochromators are fast and reliable at the same time.

Conclusion

We show that the Transcreener® ADP² FI assay is compatible with the PHERAstar FS, the CLARIOstar and the POLARstar and FLUOstar Omega.

PHERAstar® *FSX*
PHERAstar® *FS*

CLARIOstar®

Omega Series

The new Atmospheric Control Unit (ACU) for the CLARIOstar® provides versatility in long term cell-based assays

Carl Peters[1] and Tracy Worzella[2]
[1]BMG LABTECH, Cary, NC [2]Promega, Madison, WI

- **CLARIOstar with ACU maintains cell health and proliferation in untreated cells**
- **Cells can be monitored with duplexed assays**
- **Time- and dose-dependent compounds effects can be discovered directly in the cell culture**

Introduction

Analyses of cell viability and cytotoxicity are examples of cell based assays that derive great benefit from the ability to detect changes to these parameters in real time. Real-time detection allows identification of an exact moment in time when a cytotoxic or antiproliferative change occurs. When these assessments are performed with a microplate reader multiple samples and concentrations can be monitored on a single plate.

Two assays from Promega (RealTime-Glo® MT Cell Viability and the CellTox Green Cytotoxicity Assay) were measured over 72 hours. In order to maintain cell health the CLARIOstar was equipped with an Atmospheric Control Unit (ACU). This enables the detection of both time- and dose-dependent effects on cell proliferation and cytotoxicity.

Assay Principle

The RealTime-Glo® MT Cell Viability Assay is a bioluminescent assay that relies on the metabolic (MT) reducing potential of cells. NanoLuc® luciferase and cell-permeant pro-NanoLuc® substrate are added to cells in culture. Viable cells reduce the substrate which then diffuses into the medium where it is rapidly used by NanoLuc® enzyme to produce a luminescent signal proportional to viable cell number (Fig. 1).

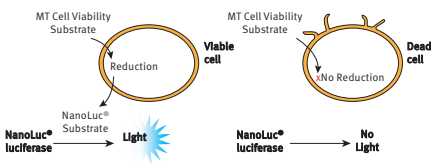


Fig. 1: The RealTime-Glo® MT Cell Viability Assay Principle.

The non-activity based CellTox™ Green Cytotoxicity Assay is comprised of a cell membrane impermeant dye that is excluded from viable cells. When the cell membrane becomes compromised, the dye enters the cell where it binds to DNA and becomes fluorescent. Fluorescent signal is proportional to the number of dead cells in culture (Fig. 2).

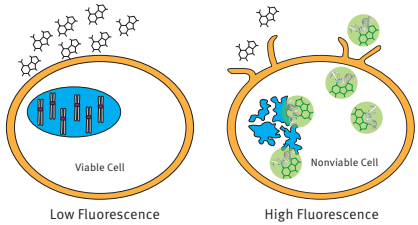


Fig. 2: The CellTox™ Green Cytotoxicity Assay Principle.

Materials & Methods

- 384-well, white, clear bottom microplates from Corning
- RealTime-Glo™ MT Cell Viability Assay from Promega
- CellTox™ Green Cytotoxicity Assay from Promega
- CLARIOstar® microplate reader from BMG LABTECH

K562 cells were plated in 384 well microplates and treated with a panel of test compounds with known effects on proliferation and cytotoxicity. The CLARIOstar with ACU was used to both incubate cells and quantify luminescence and fluorescent signal changes every hour for 72 hours.

Fluorescence instrument settings

Method:	bottom reading
Flashes per well:	50
Optic Settings:	Excitation: F: 482-16/ Emission F: 530-40
Dichroic:	LP 504
No. of cycles:	73
Cycle time:	60 min
Gain + Focus :	adjusted prior to test

Luminescence instrument settings

Measurement interval time:	1.0 s
Optic settings:	No Filter
No. of cycles:	73
Cycle time:	60 min
Gain:	3500

ACU settings

CO_2	5 %
O_2	monitoring
Target temperature	37°C

Rev. 02/2015
Keywords: **ACU Atmospheric Control Unit, Cell viability, Cytotoxicity, Multiplexing, NanoLuc luciferase**

Results & Discussion

The CLARIOstar with ACU was able to fully sustain the normal proliferation and health of untreated cells for the entire 72 hour time course (Fig. 3).

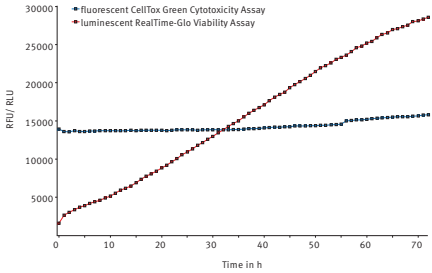


Fig. 3: Multiplexed RealTime-Glo® and CellTox™ Green assay. Average results of 10 replicates shows that cell viability increases and cytotoxicity is unchanged over 72 hours in untreated cells.

Cells treated with varying concentrations of the tyrosine kinase inhibitor bosutinib, initially exhibit proliferation although higher concentrations suppressed proliferation. For all but the lowest concentration, a change appears to occur around 25 hours and cell viability begins to decrease (Fig. 4).

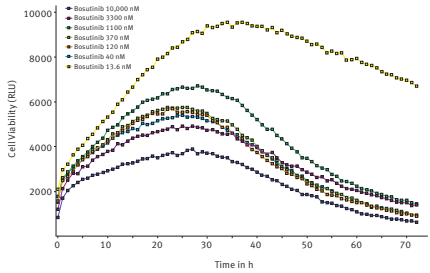


Fig. 4: Effect of varying concentrations of bosutinib on cell viability assesed using RealTime-Glo® MT Cell Viability Assay. Average results of triplicates at the indicated concentrations of bosutinib.

All concentrations of bosutinib also increased cytotoxicity to some degree. Cytotoxicity begins to increase at around 30 hours coincident with decreased viability (Fig. 5).

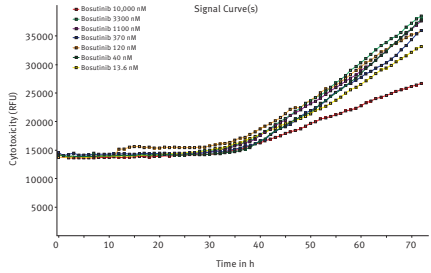


Fig. 5: Effect of varying concentrations of bosutinib on cytotoxicity assessed using CellTox™ Green Cytotoxicity Assay. Average results of triplicates at the indicated concentrations of bosutinib.

Conclusion

The CLARIOstar with ACU keeps cells happy so that long term cell-based assays can be achieved. In this case both cell viability and cytoxicity were monitored over 72 hours. Here we show the hourly assessment of the effect of the tyrosine kinase inhibitor bosutinib. The results show a clear time dependence for cytotoxicity as well as time and dose dependence for cell viability.

Atmospheric Control Unit (ACU)

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The OxiSelect Cellular Antioxidant Assay (CAA) on the FLUOstar® Omega

Hanaa Yamani[1] and Giovanni Abbenante[2]
[1]School of Applied Science Biotechnology and Environmental Biology, RMIT University, Melbourne, Australia [2]BMG LABTECH Australia

- **Determination of leaf and oil extracts**
- **Plant flavonoid quercetin used as standard substance**
- **MARS Data Analysis software offers one-click feature for whole data processing**

Introduction

Reactive Oxygen Species (ROS) are generated in the body as part of the normal metabolism process when we eat or breathe. Accumulation of abnormal levels of ROS in the body has been implicated in several diseases including diabetes, renal ischemia, atherosclerosis, cancer and ageing in general.
There is a possibility that eating foods with high levels of antioxidants can substantially contribute to an individual's health and wellbeing. In order to lend support to this theory it is necessary to be able to quantify the antioxidant potential of foods.
To this end, a suite of assays have been developed in recent years for *in vitro* antioxidant analysis of foods. These include assays such as; TEAC, FRAP, TRAP, Folin, DPPH, CUPRAC and ORAC.[1] Although these assays are able to quantitate the amount of antioxidants in raw and processed foods they do not give any information on the bioavailability of antioxidants when ingested.
In order to partly address this limitation a Cellular Antioxidant Assay (CAA) utilising human hepatocarcinoma (HEPG2) cells was first developed by Wolfe and Liu.[2] Recently the assay has also been adapted for many different cell lines.[3] In addition, a Cellular Antioxidant Assay kit (OxiSelect) is now commercially available. This application note shows results obtained with the OxiSelect kit for an anonymous plant essential oil and leaf extract.

Assay Principle

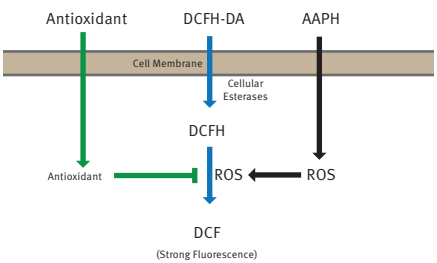

Fig. 1: Principle of the OxiSelect Cellular Antioxidant Assay.

This assay relies on the ability of live cells to allow the non-fluorescent esterified dye precursor 2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) to diffuse across the cell membrane. Once inside the cell, the dye is de-esterified to 2', 7'-Dichlorodihydrofluorescein (DCFH) by cellular esterases and remains trapped inside. The cells are washed and upon addition of the a radical initiator (AAPH) the non-fluorescent dye (DCFH) is transformed to the highly fluorescent 2', 7'-Dichlorofluorescein (DCF) (Fig. 1).

When the dye-containing cells are also incubated with an antioxidant, the free radical induced reaction leading to DCF can be prevented to a greater or lesser extent. In order to quantify the ability of an antioxidant to prevent the free radical induced reaction in the live cells, a standard curve of fluorescence vs time with varying amounts of a standard (quercetin, a strong antioxidant) is firstly constructed and the ability of various food extracts to inhibit the free radical induced reaction are compared to this standard.

Materials & Methods

- OxiSelect™ Cellular Antioxidant Activity Assay Kit (#STA-349) from Cell Biolabs, Inc.
- FLUOstar® Omega multidetection microplate reader from BMG LABTECH

The kit contains a 96-well tissue culture treated clear bottom black microplate, DCFH-DA dye, the free radical initiator (AAPH) as well as quercetin standard.

Quercetin standard curve
Preparation of quercetin standards, 2.8% radical initiator solution, and 2x dilution of the DCFH-DA stock solution was performed as described in the assay manual.

Sample Preparation
Essential oil stock concentration was 20 µl/ml of cell culture medium, from which four dilutions were prepared: 10 µl/ml, 5 µl/ml, 2.5 µl/ml, and 1.25 µl/ml. Leaf extract stock was made at 20 mg/ml cell culture medium from which 10 mg/ml, 5 mg/ml, 2.5 mg/ml, and 1.25 mg/ml solutions were prepared.

HepG2 cells at a concentration of 6 x 10^4 cells in 100 µl growth media per well were incubated for 24 hours until cells were 90% to 100% confluent. The outer wells of the microplate were left empty. After seeding, the media was removed, and cells washed gently 3 times with PBS/HBSS. All wells were then treated with 2x diluted DCFH-DA probe solution (50 µl) and with either quercetin standards or prepared samples (50 µl). The microplate was incubated for 60 minutes at 37°C. After this the liquid was removed and cells washed 3 times with PBS/HBSS, then the last wash removed and discarded. Lastly, 100 µl of free radical initiator solution was injected with the on-board injectors to all wells and the plate was read on the FLUOstar Omega at 37°C for 1 hour, collecting data every 1-5 minutes.

Rev. 05/2015
Keywords: **Antioxidant capacity, ORAC, OxiSelect, ROS, TEAC**

Controls did not contain standard or sample. Blanks consisted only of cells without addition of radical initiator.

FLUOstar Omega instrument settings

Measurement method	Fluorescence intensity
Reading mode	Plate mode, top reading
Positioning delay	0.5 s
Filters	Ex 485nm, Em 520nm
No. of flashes per well	20
No. of Cycles	12
Cycle time	300 s
Temperature	37°C

Results & Discussion

In control wells, containing only cells with dye precursor, addition of AAPH leads to a strong increase in fluorescence over time (Fig. 2).

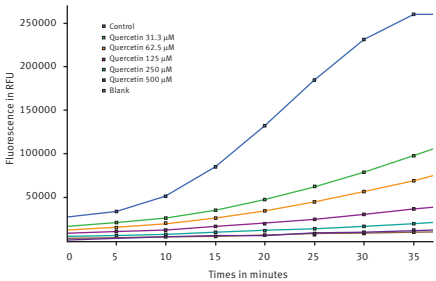

Fig. 2: Signals of controls, standards and blanks over time.

The blanks, that do not contain the radical initiator, show only a very small fluorescence increase over time, caused by ROS already present in the cells. The quercetin standard prevents the dye to be oxidized. The degree of prevention depends on the antioxidant's concentration and on its ability to cross the cell membrane and survive metabolism by the many enzymes and degradation processes in the live cell.

From the signal curves the area under the curve (AUC) is determined using the 'SUM' function in the MARS Data Analysis software. The AUC for blanks was then subtracted from all wells. CAA values were calculated by the software based on the equation:

CAA = 100 - (AUC (sample) /AUC (Control) *100).

The CAA values of the quercetin standards were used to create a 4-parameter standard curve (Fig. 3).

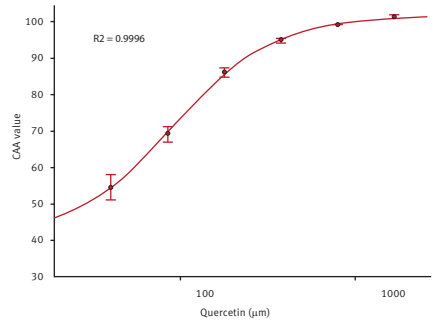

Fig. 3: CAA values dependent on quercetin concentration.

To determine the QE value of a sample, a concentration of antioxidant is chosen that falls within the concentration limits of the quercetin standard curve. The dilution factor is considered by the software. The resulting values are in µmoles of quercetin (quercetin equivalents, QE) per ml for the oil and quercetin equivalents per g for the leaf extract.

A more rigorous calculation method is to find the EC_{50} value for each antioxidant used on the plate and these compared. The method is described in detail by Wolfe.[2] The QE value determined by this method takes into account all concentrations that were measured, simultaneously. Comparison of QE values obtained with both methods is shown in Table 1. From the results it can be seen that the leaf extract has 2x more antioxidant potential than the essential oil from the same plant.

Table 1: Quercetin equivalents (QE) from essential oil and leaf extract samples.

QE value	4-parameter fit	Median effect plot
Essential oil	23.2 µmoles QE/ml	19.0 µmoles QE/ml
Leaf extract	40.7 µmoles QE/g	38.1 µmoles QE/g

Conclusion

Together with the MARS Data Analysis software, the FLUOstar Omega microplate reader can easily be used to measure cellular antioxidant potential of foods or food extracts via the OxiSelect Cellular Antioxidant Assay.

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Omega Series

Competitive bead-based fluorescence assay to quantify antibody concentration

Christine Wosnitza[1], Sebastian Giehring[1] and Franka Maurer[2]
[1]PAIA Biotech GmbH, Köln, Germany [2]BMG LABTECH GmbH, Ortenberg, Germany

- **Low sample volumes between 2-10 µl**
- **Assay time ~ 60 min for one 384-well microplate**

Introduction

The aim of cell line development is to create cell lines that are robust, show optimal growth and produce high titers of proteins such as monoclonal antibodies. To this end thousands of cell clones are seeded as single cells and are grown in the wells of a 384-well microplate. Traditionally the high performing cells of such a clone library had to be identified using methods such as Western Blot or ELISA. Both methods are very time consuming and need lots of materials as well as hands-on time.

To overcome these drawbacks the company PAIA Biotech has created a bead-based assay that allows the quantification of cell line secreted protein in a high throughput format. The technology features special 384-well plates (PAIA plates) with a pyramid-shaped transparent protrusion on a black well bottom, which allows separation of beads and detection of fluorescence in solution. Three different monoclonal antibodies were successfully measured in the CLARIOstar® microplate reader from BMG LABTECH.

Assay Principle

The assay utilizes streptavidin coated capture beads to which a defined amount of biotinylated Protein A is bound. The fluorescence marker, a FITC-labeled human antibody, competes with the analyte for binding to Protein A (Fig. 1).

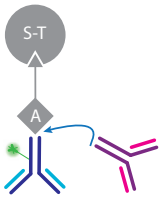

Fig. 1: Competitive assay principle.

The assay workflow is shown in Fig. 2. Capture beads are already present in the well when buffer and analyte are added (2A). Analyte capture is realized with orbital microplate shaking for 45 min (B). During the separation time of 15 min the beads bound to either analyte or the fluorescence marker will settle down (C). Finally the microplate is measured from the bottom. Only the protrusion in the middle of the well is transparent to allow the fluorescent read-out (D). The more analyte is present in the well, the more FITC-labeled antibody is replaced resulting in a high fluorescence signal.

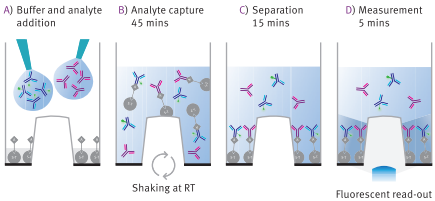

Fig. 2: Assay workflow.

Material & Methods

- Competitive human IgG Fc kit from PAIA Biotech including PAIA 384-well microplate
- Orbital plate shaker (e.g. BioShake XP from Q. Instruments)
- Monoclonal antibodies Cetuximab, Rituximab and Panitumumab were purchased from usual commercial sources
- CLARIOstar® microplate reader from BMG LABTECH

Preparation of standard curves
54 µl of PAIA mix containing assay buffer and FITC-labeled antibody is added to each well in which Protein A coupled capture beads have been pre-dispensed. 6 µl of standard at different concentrations is added using an electronic multichannel pipette. As standards three different monoclonal antibodies are used: Cetuximab (registered trade name Erbitux®), Rituximab (registered trade name MabThera®) and Panitumumab (registered trade name Vectibix®).

Shaking and sedimentation
After PAIA mix and standards are added the following shaking and sedimentation program should be applied:

- 45 min at 1800 rpm on an orbital shaker
- 5 min at 1000 rpm on orbital shaker
- 10 min without agitation

CLARIOstar instrument settings

Detection Mode:	Fluorescence
Method:	Bottom reading
Optic settings:	Monochromator presets for Fluorescein/FITC
No. of flashes:	40
Focus and Gain:	Need to be adjusted

Results & Discussion

A spectral scan was done in order to see if the typical FITC settings would be appropriate for the assay (Fig. 3).

Rev. 12/2015
Keywords: **Antibody, ELISA, IgG, PAIA, Shaking**

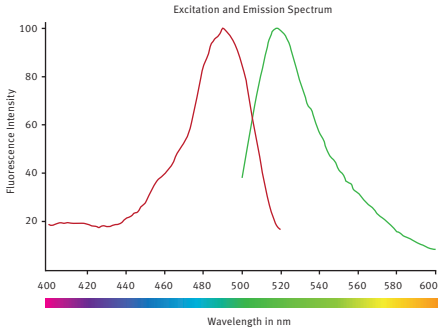

Fig. 3: CLARIOstar excitation and emission scans from wells containing labeled antibody. Excitation scan was performed between 400 and 520 nm, while the emission wavelength was 550 nm. The emission was scanned between 500 and 600 nm with a fixed excitation wavelength of 472 nm. The curves shown were scaled to 100 % intensity.

The scan has shown that the standard monochromator settings for FITC can be used for the PAIA assay (preset fluorescein/FITC). The average CV for each standard concentration with 4 replicates was 3 %.

After measuring the assay plate the data can easily be processed with the MARS Data Analysis software. A 4- or 5-parameter fit should be applied (Fig. 4).

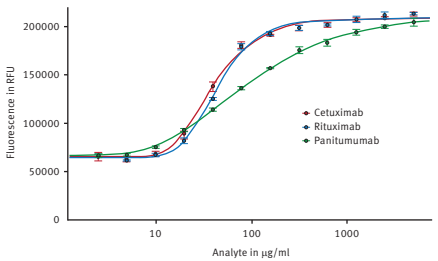

Fig. 4: 5-parameter fit standard curves for three different monoclonal antibodies added at certain concentrations to the PAIA mix. Data measured with the CLARIOstar using the LVF monochromator.

Cetuximab and Rituximab, which both are of the IgG1 type, show congruent 5-parameter fit curves indicating similar affinity to Protein A. Panitumumab is an IgG2 antibody and thus exhibits a different behaviour as can be seen by a different curve progression. Thus the assay covers different concentration ranges for IgG1 and IgG2. IgG1 can be quantified from 10-200 and IgG2 from 5-2000 µg/mL.

The PAIA assay can be measured in the CLARIOstar by either using the LVF monochromator™ or FITC filters. Table 1 shows R^2 and LoD values obtained with either configuration.

Table 1: R^2 and sensitivity comparison of monochromator and filter measurements.

	Cetuximab	Rituximab	Pantumumab
R^2 5-parameter fit			
LVF monochromator	0.9962	0.9960	0.9997
Filters	0.9973	0.9964	0.9994
*Sensitivity in µg/ml**			
LoD monochromator	10.2	13.9	6.2
LoD Filters	11.5	13.5	5.2

* LoD was calculated using the following formula:
LoD = 3 σ_0+ $\overline{y_0}$, where $\overline{y_0}$ is the mean blank signal (substance concentration is virtually 0) and σ_0 is the standard deviation for the mean blank signal.

Conclusion

The IgG PAIA assay was successfully measured with fluorescence bottom reading on the CLARIOstar microplate reader from BMG LABTECH. The special structure of the PAIA plate is compatible with all BMG LABTECH instruments capable of fluorescence intensity measurements. The competitive assay format is not limited to monoclonal antibody detection but can be extended to a variety of proteins and antibody fragments.

Lysine deacetylase activity monitored by a fluorogenic assay using the CLARIOstar®

M. Rajaee, T. Ekblad and H. Schüler
Karolinska Institutet, Department of Medical Biochemistry and Biophysics, Stockholm, Sweden

- **The CLARIOstar® monochromator functions allow convenient adaptation to commercial assay kit constituents and further optimization of assay parameters**
- **Kinetic parameters K_M, k_{cat} and V_{max} and IC_{50} value for a sirtuin enzyme inhibitor calculated**

Introduction

Protein acetylation is a universal regulatory mechanism of protein activities. Recent developments in epigenetic drug discovery have caused increasing interest in methods to study lysine deacetylation, including *in vitro* assays of lysine deacetylases.[1] Here we optimized a commercial peptidase coupled lysine deacetylase assay to establish the enzymatic properties of *Escherichia coli* CobB. CobB is a sirtuin-type enzyme that participates in the regulation of bacterial transcription, translation, metabolism, and chemotaxis.[2-4]

Assay Principle

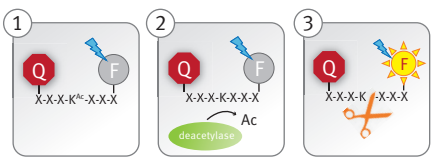

Fig. 1: Schematic illustration of the lysine deacetylase assay principle.

The CycLex SIRT1 assay kit makes use of an acetylated peptide conjugated to both a fluorophore and its quencher (panel 1). Enzymatic deacetylation of a lysine residue within the peptide (panel 2) generates a functional peptidase cleavage site. Fast subsequent cleavage of the deacetylated peptide by the peptidase separates the fluorophore from the quencher and allows fluorescent readout of the reaction (panel 3).

Materials & Methods

- 384-well black flat-bottom microplates (Greiner, #781900)
- SIRT1 fluorimetric assay kit (CycLex, CY-1151V2)
- Recombinant *Escherichia coli* CobB
- CLARIOstar microplate reader from BMG LABTECH

All standard chemicals and disposables were obtained through normal distribution channels. His6-tagged *E.coli* CobB was expressed in *E.coli* BL21(DE3) cells transfected with a pET28 expression vector, and purified using standard protocols involving immobilized metal ion chromatography (HisTrap, GE Healthcare) and size exclusion chromatography (Superdex-75, GE Healthcare). CobB lysine deacetylase activity was measured at ambient temperature (21°C) under the general conditions described in the manufacturer's protocol, with modifications as described below. Enzymatic assays were carried out using 50 nM CobB with the addition of NAD^+ and inhibitor as indicated.

Instrument settings

Detection Mode:	Fluorescence Intensity
Detection Method:	Plate Mode Kinetic
No. of cycles:	120
Cycle time (sec):	60
No. of flashes:	5
Scan mode:	orbital averaging
Scan diameter (mm):	1
Excitation:	355-10
Dichroic:	400 (autoset)
Emission:	445-10
Shaking:	8 sec before each cycle
Shaking mode:	orbital, 400 rpm

Results & Discussion

Optimization of excitation and emission parameters

Spectral scans (Fig. 2) of the fluorogenic peptide provided with the assay kit indicated that the excitation and emission settings could be improved. We used the CLARIOstar monochromators with excitation at 355/10 nm, a dichroic set at 400 nm, and emission at 445/10 nm. This resulted in the highest signal, although it did not change the Z' factor[5] which was 0.78 using either the above settings or the settings recommended by the provider.

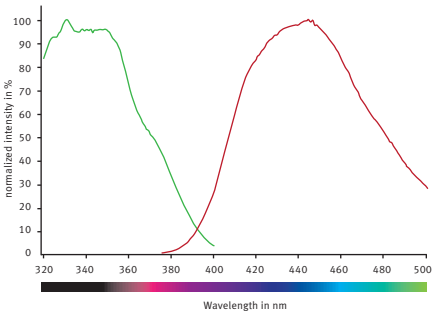

Fig. 2: Excitation and emission spectra of the fluorogenic peptide provided with the SIRT1 assay kit.

Reference curve for kinetic measurements

A dilution series of the deacetylated reference peptide provided with the assay kit was used to calibrate the measurements (not shown).

Rev. 02/2016
Keywords: **Deacetylase, Histone, K_m/V_{max}, LVF Monochromator, Sirtuin**

Basic kinetic parameters of CobB lysine deacetylase activity

We conducted CobB enzymatic assays in presence of various concentrations of NAD^+ and fluorogenic substrate peptide at the concentration recommended in the SIRT1 assay kit manual. Under our experimental conditions, we determined a K_M^{NAD+} of 71 µM (Fig. 3). We are not aware of published kinetic parameters for NAD^+ cleavage by CobB.

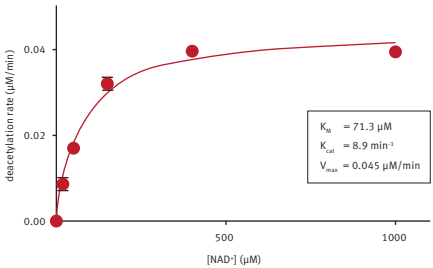

Fig. 3: Determination of the enzymatic properties of *E.coli* CobB.

Inhibition of CobB by a general sirtuin inhibitor

Ex527 is a general inhibitor of sirtuins.[6] We determined the potency of Ex527 for the inhibition of CobB activity. Assays were carried out using 70 µM NAD^+ as co-substrate and varying concentrations of Ex527 with a final DMSO concentration of 1%. The result of this analysis is shown in Fig. 4.

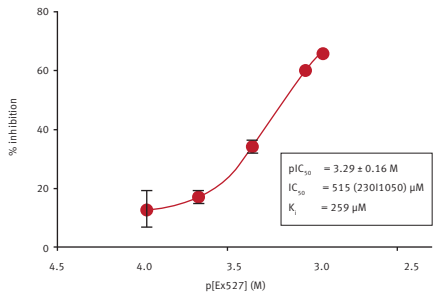

Fig. 4: Determination of the potency of Ex527 inhibition of *E.coli* CobB.

Ex527 is a weak CobB inhibitor (IC_{50} 515 µM; Ki = 259 µM determined using the Cheng-Prusoff equation). The low potency of the inhibitor, in combination with its limited solubility and the DMSO sensitivity of the enzyme, compromised the quality of the determination; the effect of Ex527 concentrations higher than 1 mM could not be measured at 1% DMSO.

Conclusion

We have used the CLARIOstar multimode microplate reader to determine the kinetic properties of the *E.coli* lysine deacetylase CobB and its inhibition by a general sirtuin inhibitor, using a commercial assay kit. Our results illustrate the general method, which should be useful for similar investigations into other lysine deacetylases and, in principle, lysine directed acetyltransferases.

Acknowledgments

The CobB expression vector was kindly provided by Dmitry Ivanov (A-Star Institute of Molecular and Cell Biology, Singapore). This work was supported by the Swedish Foundation for Strategic Research, the IngaBritt and Arne Lundbergs Research Foundation, and Karolinska Institutet.

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CLARIOstar®

Moss cells as expression system for biopharmaceuticals

Nicola Krieghoff[1], Benjamin Fode[1] and Franka Maurer[2]
[1]Greenovation Biotech GmbH, Germany [2]BMG LABTECH GmbH, Ortenberg, Germany

- **Determine total number of viable cells using autofluorescence**
- **CLARIOstar® LVF monochromator™ measures emission scans of GFP and chlorophyll**
- **Moss cells down to 500 cells per well can be detected**

Introduction

The moss *Physcomitrella patens* is a model organism in plant biology. A fast reproduction cycle, high rates of homologous recombination and the availability of efficient transformation methods of haploid protonema cells makes moss highly interesting as expression system for recombinant proteins. As a eukaryotic organism, the moss harbors all important post-translational modification features for production of biologically functional components, making it a superior choice over e.g. bacterial production systems.

In this application note we will show how to determine the total number of individualized viable moss cells per well by using the autofluorescence, represented by fluorescence from chlorophyll, and compare it with the relative number of freshly transformed, GFP-expressing cells. All fluorescence scans and intensity measurements have been performed with the CLARIOstar microplate reader from BMG LABTECH.

Materials & Methods

After generation of moss protoplasts from a bioreactor culture, the cells were transformed with a vector carrying the gene for GFP as fluorescent reporter, targeted to the nucleus. Non-transformed protoplasts were used as control. A serial dilution of these cells was done, leading to final theoretical concentrations of 225000 down to 110 cells per well. All dilutions were prepared in duplicates in black microplates with transparent bottom wells. A well filled with medium only was used as blank control. For autofluorescence and GFP measurements the CLARIOstar was set up with the following instrument protocol:

Detection mode:	Fluorescence intensity
Method:	Bottom optic used
Scan Mode:	Orbital averaging
Scan Diameter:	3 mm
No. of flashes per well:	8
Optic settings for autofluorescence	
LVF monochromator:	475-30/680-20
Optic settings for wtGFP measurements	
LVF monochromator:	385-12/510-20

Results & Discussion

Determining the relative number of transformed moss cells using chlorophyll autofluorescence and GFP

Objective of the experiment was to determine the minimum necessary number of GFP-expressing cells for measurement in the fluorescence reader. In the future, relative expression rates of recombinant proteins in different moss strains could be comparable by using a defined number of moss cells during measurements. An easy way to calculate the total number of viable cells present in the microplate well is using the autofluorescence of moss cells. Moss cells contain chlorophyll a and b. The far red emission of these pigments can be used as a tool for cell counting.

Fig. 1 shows the emission scan of untransformed moss cells as well as moss cells expressing GFP.

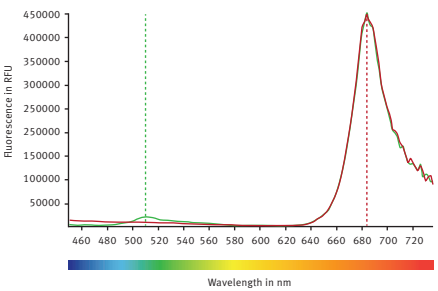

Fig. 1: CLARIOstar emission scans between 450 and 740 nm while the excitation wavelength was set at 395 nm. Mock control moss cells are represented by the red curve. Emission of GFP expressing moss cells can be followed with the green curve.

A clear peak at around 684 nm can be found in both kinds of moss cells indicating chlorophyll autofluorescence. The emission of wild type GFP at around 510 nm can only be found for GFP expressing cells. From excitation and emission scans optimal LVF monochromator chlorophyll wavelengths have been determined: excitation 475-30 and emission 680-20.

From the measurements, a standard curve could be calculated showing the relation of the chlorophyll autofluorescence to the theoretical number of moss cells (Fig. 2).

Rev. 03/2016
Keywords: **Physcomitrella patens, Moss, GFP, Chlorophyll, Autofluorescence**

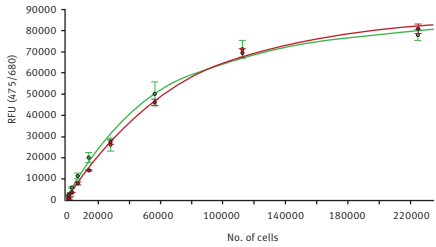

Fig. 2: Fluorescence intensity values for autofluorescence were measured with the CLARIOstar in samples of increasing number of moss cells. Green curve shows dilution of GFP expressing cells while the red curve represents serial diluted cells that do not express GFP.

The two curves are very similar, indicating that the autofluorescence is a useful tool to determine the total number of viable cells. From the standard curves, we concluded that the detection limit via autofluorescence for moss cells is around 500 cells per well.

Well scan comparison of GFP expressing cells and mock control

The distribution of protoplasts in the well may not be even. To investigate this assumption, a well scan was carried out using samples with GFP-expressing moss cells, non-transformed cells and a medium-only control.

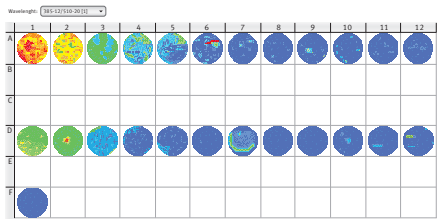

Fig. 3: CLARIOstar well scan of GFP-expressing moss cells (line A), mock control (line D) and medium only control (Well F1). Excitation wavelength 385-12 nm, emission wavelength 510-20 nm.

The results (Fig. 3) clearly demonstrate that the moss cells are indeed not distributed evenly in the wells. Some wells show aggregates, which would have negative influence on the reliability of results from a single measurement in the middle of the well. Therefore, it is recommended to use the orbital averaging function. In this mode, the measurement takes place on an orbit with definable diameter in the well. The average value of all orbit measurement points will be displayed. The use of the orbital average function will result in more stable values with decreased deviation of replicate wells.

In Fig. 3 it is shown that GFP expressing moss cells show higher fluorescence values compared to the mock control. However, also the control shows an increase in fluorescence with increasing number of cells. Thus, it can be concluded that chlorophyll is also excited by the excitation wavelength of 385 nm, and a non-transformed control is necessary for background subtraction. In this experiment, the transfection rate of GFP-construct into moss cells appeared to be around 20 %. Higher transfection rates would result in a better discrimination of GFP-expressing moss cells and the mock control and therefore in a lower total number of cells required for measurements.

Conclusion

Using the CLARIOstar microplate reader, GFP and chlorophyll emission spectra can be taken to optimize measurement settings to special medium or buffer conditions. The total number of moss cells can be determined by a standard curve using chlorophyll fluorescence emission. Moss cells down to 500 cells per well can be detected.

For analysis of GFP expressing moss cells, it is necessary to use non-transformed cells as background control, as some part of the measured fluorescence emission is related to chlorophyll.

About Greenovation

Greenovation develops plant-made next-generation therapeutics using its proprietary BryoTechnology platform. The company aims to optimize the production of highly-efficient glycoproteins for the treatment of rare diseases.

CLARIOstar®

Luminescent ABEL® antioxidant test-kit with PHOLASIN® for vitamin c type antioxidants

Bernd Hipler[1] and Jan Knight[2]
[1]BMG LABTECH GmbH, Germany [2]Knight Scientific Limited, UK

- **Measurement of ROS scavengers and other antioxidants**
- **Required sample volumes: 5 µL - 50 µL**
- **Quantitation as Ascorbate Equivalent Antioxidant Units**

Introduction

Reactive oxygen species (ROS) are essential intermediates in oxidative metabolism. Nonetheless, when generated in excess, ROS can damage cells by peroxidizing lipids and disrupting structural proteins, enzymes and nucleic acids.

Excess ROS are generated during a variety of stresses on cells including ischemia/reperfusion, exposure to ionizing and ultraviolet radiation and/or inflammation. Reactive oxygen species may contribute to inflammation and tissue damage. (e.g.: Oxidation of polyunsaturated lipids in cell membranes). Attack by a free radical on one of the carbon to carbon double bonds can lead to the formation of the corresponding lipid peroxy radical and the initiation of a chain reaction in which the final products, lipid peroxides, no longer maintain the integrity of the cell wall.

This process, lipid peroxidation, may be initiated by the hydroxyl radical OH in a reaction in which iron, as Fe^{2+}, is involved. In biological systems much attention is focused on the two free radicals, superoxide anion and hydroxyl radical.

The generation processes of ROS can be monitored using luminescence. Because of the very weak native luminescence of ROS, both luminol and lucigenin have been used in the past to give measurable signals.
However, the luminescence of these two substances is very small compared to that of Pholasin®, which allows precise analysis on very small samples.
Pholasin® is the photoprotein of the marine rockboring bioluminescent mollusc, Pholas dactylus and the Common Piddock. Pholasin® does not glow on its own, but can be switched on by free radicals and other reactive oxygen species. The capacity of samples to scavenge free radicals and other oxidants can be monitored by means of the described test kit that contains Pholasin®.

Materials & Methods

All materials were obtained through normal distribution channels from the manufacturer stated.

- BMG LABTECH microplate reader with luminescence detection and injectors
- 96-well plate, white
- Samples of unknown antioxidant capacity
- Test kit KSL-ABEL-21 M2, Knight Scientific Limited
  Website: www.knightscientific.com

In addition, consumables such as pipette tips and tubes were used as needed from various manufacturers.

Protocol for Antioxidant Test for Superoxide
Superoxide is generated instantaneously when Solution B is added to Solution A. By means of the reagent injector Solution B is injected into the microplate containing Solution A together with Pholasin®, with and without a 10 µL sample of fluid of unknown antioxidant capacity.

Into a microplate well add the following:
- 25 µL Assay Buffer (for controls) or 15 µL Assay Buffer + 10 µL sample
- 50 µL Antioxidant Pholasin®
- 100 µL Solution A

The assay is initiated when 25 µL of Solution B is injected into the microplate well, which must be in the light measuring position.

The assay is carried out using the following parameters:
Gain = 3300-3500 depending on reader, well mode, No. of intervals = 32,
Meas. Interval Time = 1 s, Total Meas. Time = 32 s, Start injection = 0 s

Standard curve for Ascorbate
Pipette into a microplate well:
50 µL Pholasin
5 µL Reconstitution and Assay buffer (R&A)
100 µL Solution A

Using the 2 built-in reagent injectors of the BMG LABTECH microplate reader the dilution series of ascorbate can be generated automatically. Injector 1 is programmed with the volumes of 1 mmol/L ascorbate solution and injector 2 with the volumes of R&A buffer as shown in Table 1.

Table 1: Volumes of ascorbate and R&A buffer added to microplate for recording a standard curve.

1 mmol/L ascorbate (µL)	R&A Buffer (µL)	Ascorbate (µmol/L) in 200 µL
0	20	0
2	18	10
4	16	20
8	12	40
12	8	60
16	4	80
20	0	100

Rev. 05/2005
Keywords: **ABEL, Antioxidant, Injectors, Pholasin, ROS, Vitamin C**

The corresponding ascorbate standard curve is shown below (Fig. 1).

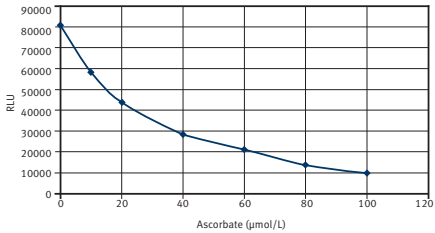

Fig. 1: Ascorbate standard curve.

Results & Discussion

A white 96-well plate loaded with the assay components and different samples was placed in the BMG LABTECH microplate reader and the reagent injector was primed with solution B. The assay was done as described above. If Pholasin® is present when the superoxide is generated, light will be emitted.
If there are antioxidants in the sample capable of scavenging superoxide, then these antioxidants will compete with Pholasin® for the superoxide and less light will be detected. Controls containing no sample are run with each assay.
The luminescence measurements with the ABEL® Antioxidant Test using different substances with unknown antioxidant capacity are shown in Figure 2.

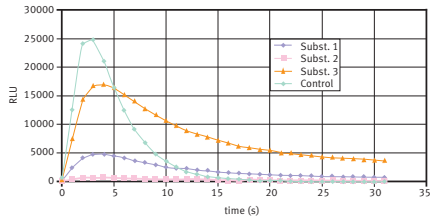

Fig. 2: Measurement of 3 different substances with ABEL® Antioxidant Test: Superoxide Assay.

These substances were prepared from natural plant extracts. Compared with the control (100%) scavenging of superoxide could be detected.
The inhibition amounted 32%, 80% and 97%, respectively. It is possible to quantify the antioxidant capacity of unknown substances by means of the ascorbate standard curve (Fig. 3).

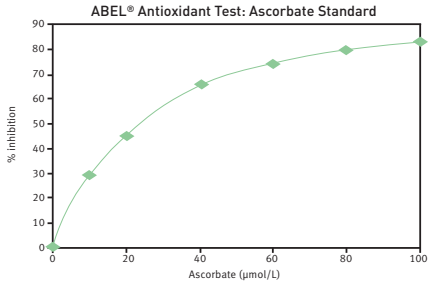

Fig. 3: Ascorbate Equivalent Antioxidant Units.

The inhibition of 80% correspond to about 77 µmol/L in the well. The Ascorbate Equivalent Antioxidant Units in the sample (10 µL) is obtained by multiplying the x value (i.e. 77 µmol/L) by the dilution factor (20) to obtain 1.5 mmol/L.

Conclusion

The ABEL® Antioxidant test kit with Pholasin® can be used successfully for measurements of substances with unknown antioxidant capacity.
With a BMG LABTECH microplate reader with luminescence detection and injectors the test can be done very fast and with a high precision and reproducibility.

Promega ENLITEN® kit performed on a BMG LABTECH microplate reader

Ronald L. Earp and Jarrett Cheek
BMG LABTECH, USA

- **ENLITEN® kit performs as expected**
- **LOD = 0.81 fmol/well**
- **BMG LABTECH microplate reader is linear through-out kit range, R^2 = 0.99595**

Introduction

The Promega ENLITEN® kit is used for the detection of ATP (adenosine 5'-triphosphate). The measurement of ATP is widely used to determine the amount of micro-organisms in food and it can be used in the quantitation of cells in tissues and serum. The ENLITEN® kit uses L/L (Luciferase/Luciferin) to rapidly quantitate ATP. The L/L catalyzes the 560 nm light-generating reaction shown below with ATP being the limiting reagent.

$$\text{ATP} + \text{D-Luciferin} + O_2 \longrightarrow$$
$$\text{Oxyluciferin} + \text{AMP} + \text{Pyrophosphate} + CO_2 + \text{Light}$$

The purpose of this experiment is to determine the analytical limit of detection (LOD). Promega indicates that the kit will detect ATP down to 10^{-16} mol or 0.1 fmol. More information about Promega's ENLITEN® kit is available on their website at www.promega.com.

Materials & Methods

All materials were obtained through normal distribution channels from the manufacturer stated.

- Promega ENLITEN® kit
- Distilled water
- BMG LABTECH microplate reader with injectors
- Microplates, White 96 well, Costar

In addition, consumables such as pipette tips and micro-centrifuge tubes were used as needed from various manufacturers.

Experimental

All reagents were prepared and the assay run according to Promega's protocol. The most important aspect of this procedure is to make absolutely certain that there is no contamination of the samples. ATP is a common compound found on almost everything and great care must be taken when handling the kit. The ATP solution was pipetted into the plate using a serial dilution with concentrations ranging from 1000 fmol/well to 0.1 amol/well. L/L solution (100 µL) was injected into each well prior to measurement using the microplate reader built-in reagent injector. The injector was cleaned with a solution of 10% bleach that was allowed to sit in the injector and tubing for 30 minutes prior to use. The tubing was then rinsed with distilled water to remove the bleach solution.

The plates were inserted into the instruments and then read in luminescence mode using the following parameters:

BMG LABTECH reader parameters:

- Emission filter: Empty (depending on reader)
- Read Mode: Well
- Gain: 3300-3500 (depending on model)
- No. of Intervals: 20
- Integration Time: 0.5 s
- Position delay: 1 s

The average value of the blank measurement was subtracted from the duplicate measurements made at each concentration and the results plotted. A linear regression was performed on the standard curve to provide the calculated values that appear in Figure 1 and Figure 2.

Results & Discussion

The performance of the ATP kit was linear over a range of 100 fmol/well to 0.195 fmol/well. A linear regression was performed on the data, which yielded an R^2 value of 0.99859. Blanks were repeatedly measured (n=20) to determine the standard deviation of a well measurement. The standard deviation of the blanks were 138 RLUs. Using these parameters the LOD, LOQ and R2 were calculated and are summarized in Table 1.

Table 1: Parameters.

Parameter	FLUOstar®	LUMIstar®
R^2	0.99589	0.99895
LOD	0.81 fmol/well	0.20 fmol/well
LOQ	2.70 fmol/well	0.67 fmol/well

The Technical Resource Manual that follows the kit states that this kit is designed to measure ATP from 10^{-11} to 10^{-16} mol. As shown by the data calculated the BMG LABTECH microplate reader performs within the stated range. However, BMG LABTECH microplate readers can read to much lower limits of detection, beyond the linearity of the Promega ENLITEN® ATP kit. The LOD for ATP using this ENLITEN® ATP kit is approximately 1 fmol per well for the FLUOstar/POLARstar series of instruments, entirely reasonable for a multifunctional microplate reader and certainly within the realm of general purpose laboratory use.
These results were taken from one representative instrument and should be taken as single instrument

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Keywords: **ATP, ENLITEN, Food contamination, Luciferase, Promega**

data only. They do not accurately reflect the reader as a whole but should give the user a reasonable expectation of performance. Results may or may not vary from instrument to instrument.

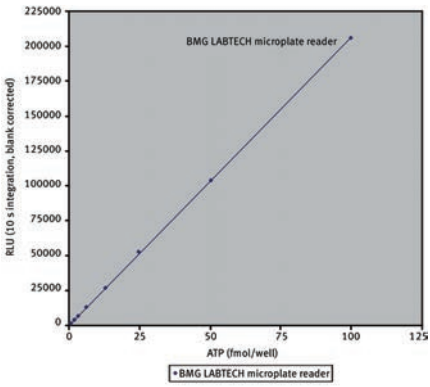


Fig. 1: Linearity data from 100 fmol/well to 0.195 fmol/well.

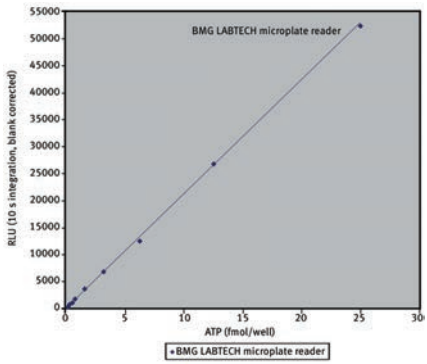


Fig. 2: Expanded range from Figure 1, 0-30 fmol/well.

PHERAstar® *FSX*
PHERAstar® *FS*

CLARIOstar®

Omega Series

Use of BMG LABTECH microplate reader to monitor the production of free radicals by leukocytes in filuted blood

Symah Lateef, Paul R. Ingram and Corinne M. Spickett
Dept. of Immunology, University of Strathclyde, Glasgow

- **Ultrasensitive analysis of free radicals**
- **Very small blood volume required (0.2µL)**
- **Simultaneous injection, shaking, and measurement**

Introduction

The ABEL® (Analysis by Emitted Light) cell activation kits for whole blood samples and isolated cells are used to analyze the real-time production of free radicals and other reactive species by leukocytes during the respiratory burst. These chemiluminescent assay kits (developed and manufactured by Knight Scientific Limited) involve the use of the light-emitting protein Pholasin®, Adjuvant-KTM (which functions to enhance the light signal during assays with whole blood), and the cell stimulants PMA (phorbol-12-myristate-13-acetate) and fMLP (n-formyl-methionyl-leucyl-phenylalanine).
Pholasin® is a photoprotein isolated and purified by Knight Scientific Ltd (KSL) from cultivated specimens of the bioluminescent mollusc, Pholas dactylus, the Common Piddock. In the assay, Pholasin® emits light in the presence of free radicals and reactive species produced by the NADPH oxidase system of stimulated leukocytes. The assay is ultrasensitive, using 20 µL of blood diluted 1:100 (the equivalent of 0.2 µL whole blood) in a final volume of 200 µL. Light is measured in a BMG LABTECH microplate reader.

Materials & Methods

The materials were purchased from the following manufacturers:

- ABEL® cell activation test kit for whole blood or isolated cells with Pholasin® and Adjuvant-KTM, Knight Scientific Ltd.
- BMG LABTECH Microplate Reader with reagent injectors.

Blood samples were drawn into 5 mL evacuated vials containing EDTA and analyzed on the day of collection. All other reagents required were provided with the kit and were prepared following the instructions supplied with the kit. The microplate was prepared with the reagents and diluted whole blood samples according to the detailed protocol provided. Plasma was separated from an aliquot of each sample and assayed as the control for the corresponding whole blood sample.
All samples were assayed in triplicate. Prior to the preparation of the microplate, the injectors were primed with the cell stimulants: injector 1 with PMA and injector 2 with fMLP. The temperature was also set to 37°C. Once ready, the plate was inserted into the reader to allow the reagents and samples to reach 37°C before measuring the luminescence. During this time, the instrument was set up with the following parameters:

With PMA:

Mode:	Plate
Gain:	3300-3500 (depending on reader)
Meas.	time/well: 1 s
Positioning	delay: 0.1 s
Shaking:	Orbital, during measurement
Cycle time:	This parameter is automatically calculated from the number of samples being analyzed, i.e. the number of wells being measured, and is adjusted appropriately for the number of samples being assayed on a specific 96 well plate.
Number of Cycles:	This is calculated from the total assay time (s) within plate mode divided by cycle time (s).

PMA was injected after 60 seconds, therefore the cycle at which injection was to occur was calculated according to the cycle time of each particular assay.

With fMLP:

Mode:	Well
Gain:	3300-3500 (depending on reader)
No. of intervals:	200
Measurement time/well:	2 s/well
Positioning delay:	0.1 s
Injection 2 start time:	60 s
Shaking:	Orbital, during measurement

Upon completion of the experiments, the injectors were washed with water, 10 mM NaOCl(aq), water, 50% ethanol, and a final rinse with water. Thorough washing was very important for correct maintenance of the injectors.

Results & Discussion

The luminescence was measured as relative light units per second (RLU/sec). The increase in measured emission of light illustrated the presence of free radicals released by activated leukocytes during the respiratory burst following stimulation. With fMLP, which activates the NADPH oxidase by binding to receptors on the plasma membrane, the respiratory burst commenced almost immediately (within approx. 5 s) as can be seen from the increase in the signal peak in Figure 1.

Rev. 05/2007
Keywords: **ABEL, Free radicals, Injectors, Leukocytes, Pholasin, ROS**

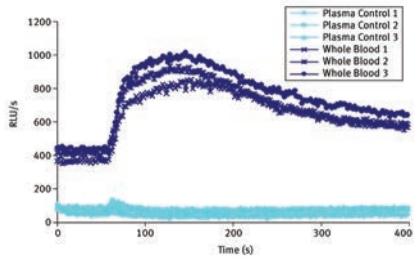


Fig. 1: fMLP Stimulation of Plasma and Leukocytes in Whole Blood.

The response started to tail off after 2-3 minutes. A longer lag time was observed in response to PMA (Figure 2) which enters the cell and activates the NADPH oxidase system via direct activation of protein kinase C. With PMA the NADPH oxidase system of both the plasma membrane and the secondary granules of leukocytes is activated. Figure 2 shows the results of measuring luminescence for a total time of 45 minutes, with PMA injection after the initial minute of measuring time. The signal increased to a maximum level and then became constant. Plasma was used as a control to measure baseline luminescence, as it contains no cells.

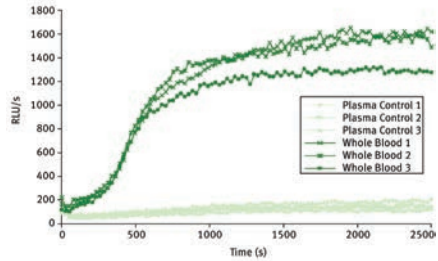


Fig. 2: PMA Stimulation of Plasma and Leukocytes in Whole Blood.

Using the BMG LABTECH microplate reader to measure the light emitted in these assays had a number of advantages. The sensitivity of detection was more than adequate to detect free radicals produced from leukocytes in 0.2 µL of blood (containing on average 500 neutrophils).

The automated injectors provided the ability to inject the cell stimulants and measure luminescence simultaneously, which was of greatest advantage when injecting fMLP. Stimulation with fMLP is initiated by binding to receptors resulting in the rapid production of superoxide (2-5 s lag time), which is detected by a rapid increase in light emission from Pholasin®. The ability to maintain the temperature control allowed the assays to be carried out at physiological temperature. Of particular value in the whole blood assay was the ability to measure light while the microplate was being shaken; this feature ensured that the blood cells along with all the reagents were in suspension during the assay. Temperature control and shaking were important factors for obtaining good reproducibility and ensuring that any differences in results observed were due to inherent differences in the activity of the leukocytes.

The use of an ATP bioluminescence assay to quantify cell cytotoxicity

Bernd Hipler
BMG LABTECH, Germany

- **Luciferase/luciferin reaction used to measure the amount of ATP in cells**
- **ATP bioluminescence assay offers high sensitivity and a wide dynamic range**

Introduction

The quantification of viable cells in culture is a very important problem. However, the technique becomes much more valuable when the effect of growth regulatory substances and also cytotoxic agents are under study. Measurement of viable cell numbers in culture is a critical aspect in the functional in vitro bioassay of a wide range of growth factors. The nucleotide adenosine triphosphate (ATP) plays an important role in energy exchange in biological systems. It serves as the principal immediate donor of energy and is present in all meta-bolically active cells. ATP has been used as a tool for the functional integrity of living cells since all cells require ATP to remain alive and carry out their specialized function. Most ATP is found within living cells and links catabolic and anabolic processes. Cell injury or oxygen/substrate depletion results in a rapid decrease in cytoplasmatic ATP. Measurement of ATP is therefore fundamental to the study of living processes. A lot of methods have been used for ATP determination, but far the most successful technique is the bioluminescent method, because of its sensitivity and the wide dynamic range. ATP bioluminescence has been used for determining levels of ATP in a number of different cell types.

Assay Principle

The reaction is catalyzed by the enzyme luciferase obtained from the firefly (Photinus pyralis). The $MgATP^{2-}$ converts the luciferin into a form which is capable of being catalytically oxidized by the luciferase in a high quantum yield chemiluminescent reaction, according to the following equation:

$$\text{ATP + D-Luciferin + } O_2 \xrightarrow{\text{luciferase}} \text{Oxyluciferin + AMP + PPi + Light}$$

Under optimum conditions, light intensity is lineary related to ATP concentration. Cellular ATP can be measured by direct lysis of the cells with suitable detergent, the released ATP is then free to react with the luciferin-luciferase and leading to light emission at 562 nm.
In this study ATP bioluminescence was used to determine whether there is a linear relationship between the number of HaCaT cells present in the culture and measured luminescence. It was attempted to use ATP-bioluminescence as a measure of proliferation and/or cell cytotoxicity depending of two different substances and avoid the use of radio-isotopes. These investigations have been carried out using the keratinocyte cell line HaCaT.

Materials & Methods

Reagents and instrumentation
HaCaT cells, propagated in 75 square cm tissue culture Hasks with weekly passage in DMEM (Seromed, FG 0435, Biochrom KG, Germany) containing 10% fetal calf serum and 1% antibiotic/antimycotic solution at 37°C and 6% CO_2. Cells were seeded in microtitre plates (Greiner 96-well plates) at a density of 5000 cells.
One day after plating in the serum-free medium (Gibco-Defined Keratinocyte-SFM), in SFM supplemented hyaluronic acid, hyalogran or medium was added over a period of 24h or 48h later, cell proliferation was measured.

ATP bioluminescence
ATP releasing agent (Somalyze), Tris-acetate buffer, ATP-monitoring reagent (firefly luciferase, D-luciferin) and ATP-standard (EG+G. Wallac, Turku, Finland) are needed for the assay.
100 µL of ATP releasing agent was added to each well of a 96 well microtitre tissue culture plate (Greiner, Germany), containing 100 µL of HaCaT cells, and incubated for 5 minutes at room temperature. Then 180 µL of the cell lysate were added to wells of a 96 well white opaque microtitre plate.
This plate was loaded into a BMG LABTECH microplate reader and 20 µL of the luciferin-luciferase reagent (reconstituted with 10 mL of 0.01.M Tris-acetate buffer, 2 nM EDTA buffer ph 7.75) were added to each well by means of an injection pump, and the sample were measured.
Immediately after addition of luciferin-luciferase luminescence was monitored over a period of 10 s (Fast kinetic method). Light output was given as the integral relative light units (RLUs). ATP measurements were carried out at room temperature.

Results & Discussion

Cell numbers and the degree of cell proliferation/ cytotoxicity were determinded by ATP bioluminescence and in comparison to a conventional method, the staining with dye Hoechst 33342.
HaCaT cells were initially used to determine whether there was a correlation between cell numbers and measured ATP. The standard curve of ATP was determinded in several groups of experiments (Fig. 1) ($r = 0.998$; $p < 0.0001$). From figure 2 it can be seen that after incubation of increasing cell numbers with ATP releasing agent (100 µL) for 5 min at room temperature an increase of released ATP ($r = 0.99$; $p < 0.0001$) can be observed.

Rev. 05/2007
Keywords: **ATP, Cell viability, Cytotoxicity, HaCaT cells**

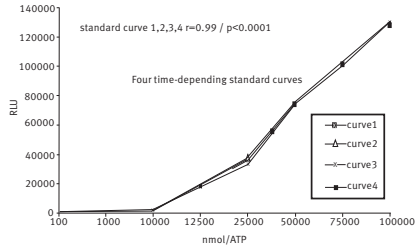


Fig. 1: Determination of ATP-standard curves for 24h incubation with several substances.

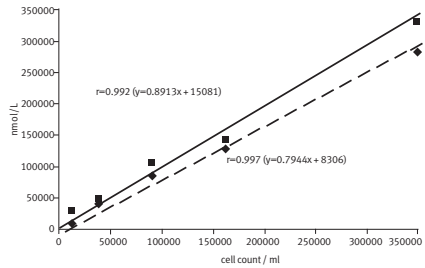


Fig. 2: Correlation between cell counts.

The correlation was determined from comparison of cell numbers with ATP-bioluminescence readings for each data point and Spearman's rank correlation was determined for the group as whole. Complete medium cultured in the absence of cells served as a control and contained no detectable ATP. Incubation with 100 µL releasing agent for 5 min at room temperature was optimal for determining the amount of ATP in cultured HaCaT cells. In figure 3 and 4 the correlation between the cell numbers and the ATP concentrations of several experiments were shown after 24h and 48h, respectively.

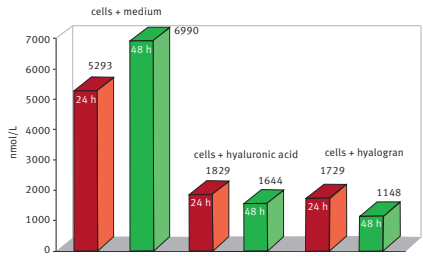


Fig. 3: ATP determination in HaCaT cells after 24 h or 48 h incubation with serveral substances.

The data show that with HaCaT cell line tested there were significant correlation between increased cell number and ATP measured by the luciferin-luciferase reaction. In order to show that ATP is an indicator of proliferation and/or cell cytotoxicity the assay was carried out under addition of two different substances. These substanced are hyaluronic acid and hyalogran. Hyaluronic acid (HA) is a major component of extra-cellular matrix, distibuted ubiquitously, with the highest concentration in the soft connective tissue.
Especially the influence of hyaluronic acid is in good agreement with the fact that high molecular weight HA in high concentrations inhibits movement, adherence and phagocytosis of several cell types, while low molecular weight HA at low concentrations stimulates cellular migration, proliferation and phagocytosis.

Conclusion

This study confirmed that the concentration of ATP in the HaCaT cell line can be determined by luciferin-luciferase bioluminescence assay, and also that the monitoring of ATP bioluminescence can be used for the measurement of proliferation and/or cytotoxicity.

Lonza's MycoAlert™ assay on a BMG LABTECH plate reader

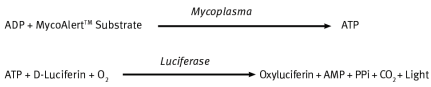
Anne Cox and Anthony Pitt
Cambrex Bio Science belongs now to Lonza

- **Detection of mycoplasma in as little as 20 minutes**
- **Rapid, simple and highly sensitive bioluminescent assay**
- **Detection down to 10 CFU/mL**

Introduction

Mycoplasma are a serious contaminant of cell cultures, they are resistant to antibiotics such as penicillin and can alter a wide range of cellular functions while their small size means that even very high levels of contamination can go completely unnoticed. Mycoplasma contaminations of cell cultures have previously been difficult to detect easily and quickly requiring lengthy culturing procedures or subjective staining methods. Lonza has developed MycoAlert™, a rapid, simple and highly sensitive bio-luminescence based assay that allows the detection of mycoplasma in as little as 20 minutes.

By making use of enzymes peculiar to mycoplasma metabolism, the MycoAlert™ Substrate catalyses the formation of ATP from ADP, which can then be detected using the highly sensitive bioluminescent luciferin/luciferase reaction utilised in the MycoAlert™ Reagent as shown in the reaction equation below.



By measuring an increase in ATP over that of background ATP, a ratio can be calculated that when above 1 is indicative of the presence of mycoplasma in the sample (usually cell culture supernatant).

The biochemical activity detected by MycoAlert™ in mycoplasma is conserved across species allowing the assay to detect far more than just the 6 common species (*M. arginini, M. salivarium, M. fermentans, M. hyorhinis, M. orale and A. laidlawii*) found to routinely contaminate cell cultures. MycoAlert™ allows very low levels of ATP production to be detected and therefore very low levels of infection. The assay can typically detect 50 CFU per mL or less although specific tests with *M. hyorhinis, M. orale* and *A. laidlawii* with independently enumerated samples have shown detection down to 10 CFU per mL.

Any luminometer used with the MycoAlert™ assay has to be sensitive enough to cope with low levels of ATP detection when few or no mycoplasma are present. The BMG LABTECH microplate reader equipped with luminescence can do this, especially when care has been taken to use correct settings.

The purpose of this application note is to show how this is done using the MycoAlert™ Assay Control Set that is supplied by Lonza.

Materials & Methods

All materials were obtained through normal distribution channels from the manufacturer stated.

- MycoAlert™ Mycoplasma Detection Kit, Lonza
- MycoAlert™ Assay Control Set, Lonza
- BMG LABTECH microplate reader
- Microplates, white 96-well, Porvair Sciences

All reagents were prepared and the assay run as stated in the MycoAlert™ kit inserts. In this instance, manual addition of the reagents rather than by automated injectors was used.

MycoAlert™ Assay Control (a purified enzyme used as a positive control) was diluted serially 1 in 2 with MycoAlert™ Assay Buffer (samples were kept on ice until used). The MycoAlert™ Assay reagent was added to enable the detection of background ATP in the sample and measured with the BMG LABTECH plate reader after a 5 minute incubation at ambient temperature (reading A) as shown in figure 1. Once read the cycle was paused and MycoAlert™ Assay Substrate added, and the cycle resumed to allow a ten minute incubation at ambient temperature prior to reading the luminescence (reading B).

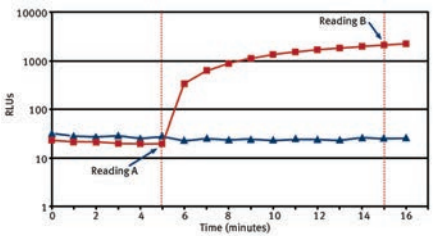


Fig. 1: The kinetic profile of a MycoAlert™ reaction where blue (▲) is a mycoplasma free sample, and red (■) is a mycoplasma positive sample (K562 cell culture infected with *M. hyorhinis*). The profile shows the rapid production of ATP by the mycoplasma positive sample after the addition of the MycoAlert™ Substrate.

Instrument settings

Read mode:	Plate
Gain:	3300-3500 depending on reader
Optics:	Top Optics
Number of cycles:	2 with pause and 10 minute delay
Integration time:	1 second
Position Delay:	Default 0.1 second

Rev. 10/2004
Keywords: **Cell culture, CFU colony forming units, Contamination, MycoAlert, Mycoplasma**

Results & Discussion

The MycoAlert ratio was calculated by the final reading B/background reading A, a positive ratio being any value greater than 1.

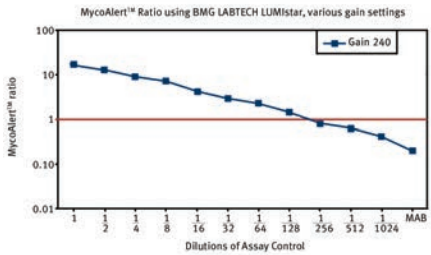


Fig. 2: MycoAlert™ ratio control dilutions.

M. orale dilutions of known CFU are shown in figure 3.

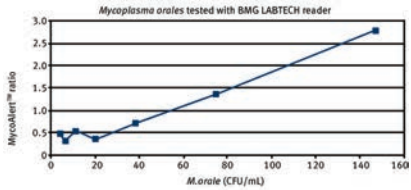


Fig. 3: 50 CFU/mL can be detected using the BMG LABTECH reader.

These results were taken from one representative instrument only and should be taken as single measurement data, giving the user an expectation of the sensitive performance of BMG LABTECH readers in conjunction with the MycoAlert™ Kit, but not absolute criteria.

MycoAlert is a trademark of Lonza

MycoAlert™ Ready Device Lonza

Lonza's PKLight™ protein kinase assay on the PHERAstar® *FS* plate reader

Andrew Paine and Lee Walker
Cambrex BioScience belongs now to Lonza

- **Screening of potentially all protein kinases in formats up to 1536**
- **Costeffective: No need of specific antibodies or radioactive beads**
- **Simple and robust assay with Z' > 0.8**

Introduction

Protein kinases and their ability to phosphorylate proteins play key roles in the signal transduction pathways of many diseases such as cancer, arthritis and diabetes. The importance of protein kinases makes them common targets for many High Throughput Screening (HTS) departments within the pharmaceutical industry. Current screening technologies employ the use of phosphostate specific antibodies or radioactive beads.

Lonza has developed PKLight™, a non-radioactive, homogeneous, robust and simple assay suitable for the screening of potentially all protein kinases in 96-, 384- and 1536-well formats. This technology utilises Luciferase bioluminescence to measure ATP consumption as a result of kinase phosphorylation of the target substrate. The assay can be easily optimised for each kinase/substrate pair to produce rapid, quality data suitable for IC_{50} determination of screen compounds.

This technology does not require antibodies, radioactive beads, radiolabelled ATP or specifically modified substrate sequences. The signal is glow luminescence with a half life greater than 2 hours, which is detected using a luminometer, in our case the multifunctional BMG LABTECH PHERAstar® *FS* plate reader. In this application note we use the Ser/Thr Kinase, cAMP dependant protein kinase (PKA) to demonstrate the assay.

Assay Principle

During a kinase reaction, the level of free ATP in the reaction mixture decreases as the γ-phosphate is transferred from the ATP molecule to the kinase substrate. This drop in free ATP can then be accurately measured. The bioluminescent reaction is catalysed by the firefly luciferase enzyme and provides speed, sensitivity and convenience. The reagent contains Luciferin and Luciferase, which emits a stable light signal, the intensity of which is proportional to the concentration of ATP (figure 1).
Assay Principle: The amount of free ATP added to the reaction mixture which is consumed during the kinase reaction can be accurately measured using the patented bioluminescent Luciferase kinase reagents from Lonza. A stable light signal is emitted that has an intensity which is proportional to the concentration of ATP present. As the kinase reaction progresses the ATP concentration drops and the light emitted becomes less intense.

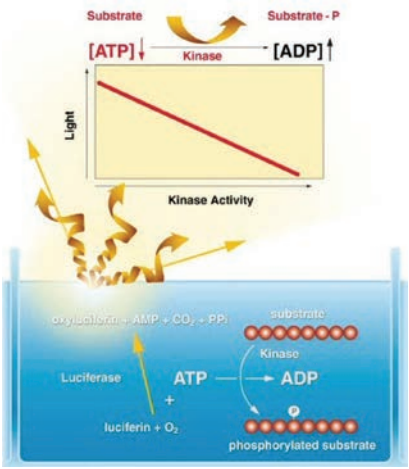


Fig. 1: Schematic assay principle.

Materials & Methods

All materials were obtained through normal distribution channels from the manufacturer stated.

- Lonza PKLight™ kit
- PHERAstar *FS*, BMG LABTECH, Germany
- Microplates, white 384-well, Greiner, Germany
- ATP, BSA, Sigma UK
- PKA, Kemptide, H-89 dihydrochloride, Calbiochem®

The Lonza Kinase Assay reagents were used to investigate the inhibition by H-89 dihydrochloride of cAMP dependant protein kinase (PKA) phosphorylation of Kemptide substrate. Enzyme, Substrate, ATP and H-89 dihydrochloride were diluted to working concentrations using the same PKA assay buffer consisting of 40 mM Tris-HCl (pH 7.5); 20 mM $MgCl_2$, and 0.1 mg/mL BSA in purified water.

ATP Detection
ATP was diluted in PKA assay buffer to give a concentration range of 0 – 12.5 µM and added into a Greiner 384-well plate. ATP detection reagent was added and read after 1 minute using a 1 second integration time in luminescence mode on the PHERAstar *FS*.

PKA Activity/Inhibition
Using white 384-well plates, 5 µL of PKA, 5 µL Kemptide, 5 µL of ATP and 5 µL of Inhibitor (H-89 dihydrochloride) or PKA assay buffer was added to each well. Giving a total assay volume of 20 µL per well. The reaction mixture was incubated for 20 minutes at room temperature.

Rev. 08/2005
Keywords: **1536-well, ATP, cAMP, PKLight, Protein Kinase A PKA**

The remaining amount of ATP was determined by adding 10 µL of the ATP detection reagent to the well and incubating for 10 minutes at room temperature. The 384-well plate was read on the PHERAstar *FS* in luminescence mode using 1 second integration.

Results & Discussion

The PKLight™ Protein Kinase Assay as shown in figure 2 can detect low levels of ATP and has exceptional linearity (R^2 >0.99) over the range used on the PHERAstar *FS*.

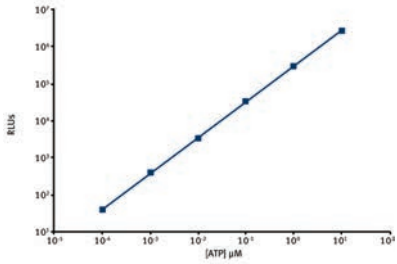


Fig. 2: Measurement of ATP (100 pM to 12.5 µM) using PKLight™ reagents and protocol. The graph demonstrates that the signal is linear over the full ATP concentration range used (R^2 values ≥ 0.99).

As shown in figures 3 and 4 the PKLight™ assay can accurately determine kinase activity and inhibition, giving reliable IC_{50} data and clean hits.

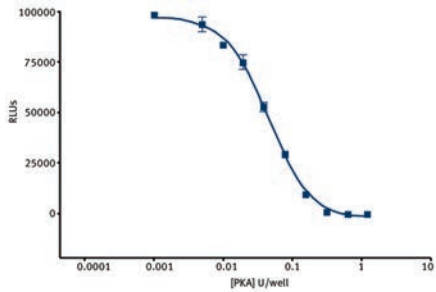


Fig. 3: PKA activity was measured from 0.001 to 1 Units/well using 1 µM ATP and 5 µM Kemptide substrate in a final assay lume of 20 µL per well.

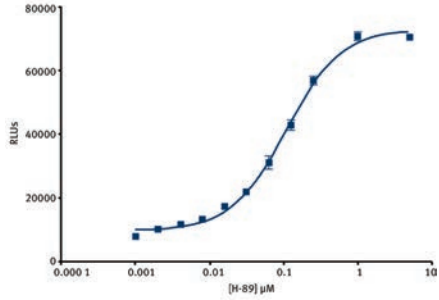


Fig. 4: Inhibition of PKA activity with H-89 dihydrochloride (0.001 - 5 µM) using 0.005 Units/well PKA, 5 µM Kemptide, 1 µM ATP incubated for 20 mins at room temp. Performed in 384 format, 20 µL total reaction volume using PKLight buffer A protocol. IC_{50} = 0.11 µM.

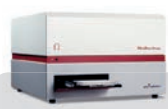
Conclusion

Using the cAMP Dependant Protein Kinase (PKA) and Kemptide substrate as a model we have demonstrated the usefulness of using the Lonza kinase reagents to measure kinase activity and inhibition.

This method is very simple to develop and run and can potentially be applied to any ATP-dependent protein kinase and target substrate. We have proved the assay to be robust with Z' values greater than 0.8 exhibited and reproducible and sensitive enough to determine low potency inhibition. The assay can be easily miniaturised down to 384-well plate formats with the potential to go beyond.

PHERAstar® *FSX*
PHERAstar® *FS*

CLARIOstar®



Omega Series

HitHunter® cAMP XS+ assay for GPCR screening using the PHERAstar® *FS*

Lindy Kauffman and Sherrylyn De La Llera
DiscoverX Corporation, Fremont, CA 94538, USA

- **Directly assess either Gi- or Gs- coupled GPCRs in cells**
- **Chemiluminescence detection reduces interference from fluorescent compounds**
- **Assay miniaturization up to 1536-well plate format**

Introduction

G protein-coupled receptors (GPCRs) make up a major family of cell surface receptors which mediate intercellular communication and GPCRs are a rich source of "druggable" targets. It has been estimated that 50% of prescription drugs interact with GPCRs. With the increasing popularity of functional assays for high throughput screening, an increasing need arises for robust second messenger assays that reflect GPCR activation and that are readily amenable for miniaturization. GPCRs that modulate adenylyl cyclase activity upon agonist stimulation and, consequently, cellular cyclic adenosine monophosphate (cAMP) levels, via the G protein Gs or Gi, form a subset of therapeutic targets. cAMP acts at several downstream targets including ion channels, kinases that modulate gene transcription, and cell metabolism. Changes in the intracellular cAMP levels correlate with GPCR activation and therefore measurement of cAMP is a simple functional assay frequently utilized in the HTS laboratory.

DiscoverX supplies several homogenous cAMP assays based on chemiluminescence to fit the various needs of the HTS customer.
HitHunter® cAMP XS+, is specifically targeted to provide enhanced performance and usability. In this application note we describe a cAMP assay based on the enzyme fragmentation complementation (EFC) of ß-galactosidase(ß-gal) measured on BMG LABTECH's PHERAstar® *FS* multi-mode HTS plate reader.

Assay Principle

The DiscoverX EFC technology is based on two fragments of *E. coli* ß-galactosidase (ß-gal), a large protein fragment (enzyme acceptor, EA) and a small peptide fragment (enzyme donor, ED). Separately these fragments are inactive, but in solution they rapidly complement (recombine) to form active ß-gal enzyme which can hydrolyze substrate to produce luminescence. In this assay cAMP from cell lysates and ED-labeled cAMP (ED-cAMP) compete for antibody binding sites. Steric hindrance of EFC occurs when the antibody binds the ED-conjugated cAMP such that little or no active ß-gal is formed. However, free cAMP generated by the cell can competitively displace the ED-conjugated cAMP from the antibody and allows the ED-conjugate to freely complement EA and thus generate a signal directly proportional to the amount of cAMP present in the cell (figure 1).

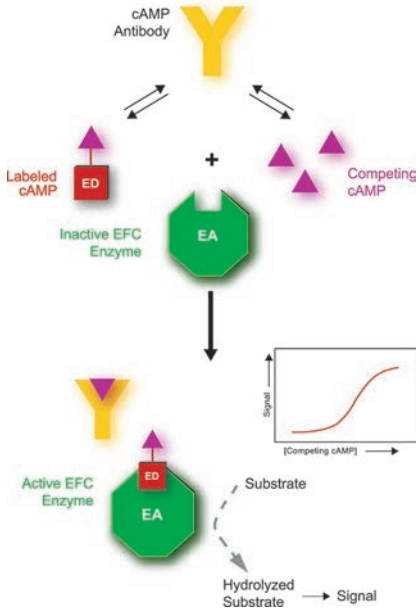


Fig. 1: Schematic principle of the HitHunter® cAMP XS+ assay.

Materials & Methods

BMG LABTECH's PHERAstar *FS* combines rapid plate reading necessary for HTS with the enhanced performance and sensitivity needed to read small liquid volumes. The PHERAstar *FS* was run in luminescence mode for the monitoring of the HitHunter® cAMP XS+ demo kit (DiscoverX Corporation) containing following reagents:

1) cAMP XS+ Lysis Buffer
2) cAMP XS+ EA Reagent
3) cAMP XS+ ED Reagent
4) cAMP XS+ Antibody Reagent
5) cAMP XS+ Standard (250 µM)
6a) Galacton-Star®
6b) Emerald-II™

HitHunter® cAMP XS+ is a single set of reagents which can be used in two different protocols, depending on the needs of the end user.

Rev. 01/2006
Keywords: **1536-well, cAMP, DiscoverX, GPCR, HitHunter**

- In the three reagent addition protocol cells are plated and induced in PBS or media, and cell induction is followed by three reagent additions and two incubation steps.
- In the two reagent addition protocol, cells are plated and induced in a diluted antibody solution, and cell induction is followed by two reagent additions and two incubation steps.

The table below outlines the volumes and procedure for the cAMP XS+ three reagent addition protocol standard curve in a 384-well plate format.

Table 1: cAMP XS+ Three Reagent Addition Protocol: Standard curve.

384-well Plate	cAMP XS+ Three Reagent Addition Protocol: Standard Curve
Step 1	10 µL of diluted standard 5 µL PBS
Step 2	5 µL Antibody Reagent
Step 3	20 µL ED/Lysis/CL Working Solution
Step 4	Incubate 1 hour at room temperature
Step 5	20 µL EA Reagent
Step 6	Incubate at least 1 hour at room temperature, then read luminescence signal on PHERAstar *FS*

The standard curve for DiscoverX's cAMP XS+ kit was run according to the package insert protocol in white 384-well plates (COSTAR) with 60 µL total assay volume. Chemiluminescence was read on the PHERAstar *FS* two hours after the addition of the last reagent using a measurement time of 1 second.

Results & Discussion

DiscoverX's HitHunter® cAMP XS+ assay was prepared in 384-well format and standard curves were run on BMG LABTECH's PHERAstar *FS* in luminescence mode. Cyclic AMP standard detection reagents were added according to the assay protocols (two and three reagent addition protocols) and chemiluminescence for both was read two hours after the addition of the last reagent (figure 2). EC_{50} values were calculated using Graphpad Prism software.

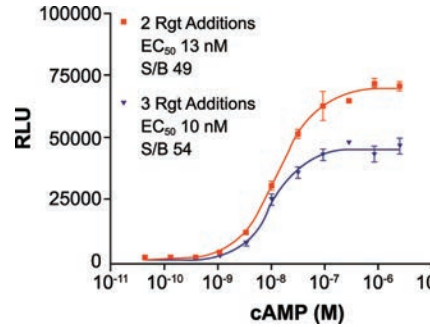


Fig. 2: Standard curve data for HitHunter® cAMP+ run in the two and three reagent addition protocol in 384-well plate format.

Conclusion

GPCRs are important targets in the HTS drug discovery approach and GPCR signalling can be examined by direct quantitation of cAMP by applying the HitHunter® cAMP XS+ kit. Excellent results were achieved on the PHERAstar *FS* multimode microplate reader which is designed to read all leading HTS detection modes in all formats up to 1536. The high degree of sensitivity, easy-to-use software, robust hardware, and optimized detection systems make the PHERAstar *FS* ideal for GPCR analyses in the high-throughput assay environment.

The PHERAstar *FS* was run in luminescence mode for the HitHunter® cAMP assay which uses enzyme fragment complementation (EFC) for sensitive detection and fewer false positives. This assay is a useful tool for those customers screening difficult targets with low basal levels of cAMP (cell lysate, tissue, or serum), or low levels of cAMP response. The chemiluminescence signal is robust and assures minimal interference from library compounds. The stable signal allows flexibility in read time, i.e. the assay can be read the same day or after an overnight incubation.

While there are several cAMP assays currently available, most of them are not scalable for miniaturization into the 1,536-well format employed for automated high throughput screening of large chemical libraries. DiscoverX offers several different configurations of this assay based on different applications, readouts, sample and cell types. For more information on DiscoverX assays please refer to the web site: www.discoverx. com

PHERAstar® *FSX*
PHERAstar® *FS*

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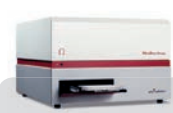


Omega Series

Study of GPCR pharmacology using the DiscoverX® HitHunter® cAMP HS assay on a BMG LABTECH microplate reader

Julie M.-N. Rainard, Stewart E. Mireylees and Mark G. Darlison
School of Biomedical and Natural Sciences, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK

- **The assay directly measures the activity of G-protein coupled receptors (GPCRs), coupled to either Gi or Gs proteins**
- **The assay is sensitive and can detect low levels of cAMP (ideal for cell-lines expressing endogenous receptors)**
- **The assay can be used on a BMG LABTECH microplate reader**

Introduction

G-protein coupled receptors (GPCRs) are cell surface receptors, which represent the most predominant drug targets. Following stimulation of these receptors, intracellular signalling pathways are activated and this leads to a decrease (coupling to a G_i protein) or increase (via G_s or G_q proteins) in the production of intracellular second messengers. The common way of determining the activity of compounds is by measuring the cellular formation of second messengers such as cAMP and calcium.

The HitHunter® cAMP High Sensitivity (HS) assay is able to measure low cAMP levels and is, therefore, particularly suitable for cell-lines that endogenously express receptors at a level much lower than in transfected cells overexpressing a cloned GPCR.
HitHunter® cAMP assays are in vitro-based competitive immunoassays that rely on enzyme fragment complementation technology (EFC, Fig. 1).

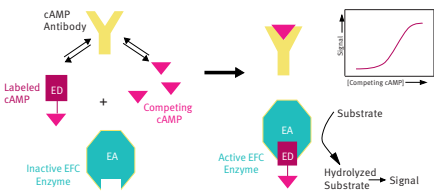


Fig. 1: The Hit Hunter® cAMP assay principle.

Free cAMP molecules from cell lysates compete for antibody binding with a labelled enzyme donor (ED)-cAMP conjugate, which contains a small peptide fragment of ß-galactosidase. In the absence of free cAMP, the ED-cAMP conjugates are captured by the cAMP-specific antibody and are unavailable for complementation with the enzyme acceptor (EA), resulting in a low signal. In the presence of free cAMP, antibody sites are occupied, allowing the ED-cAMP conjugate to complement with EA, forming an active ß-galactosidase enzyme; substrate hydrolysis by this enzyme produces a chemiluminescent signal. The signal generated is in direct proportion to the amount of free cAMP bound by the antibody. Any luminescence reader used with the HitHunter® cAMP HS assay has to be sensitive enough to detect small changes in cAMP levels. BMG LABTECH microplate readers have this required sensitivity.

The purpose of this Application Note is to detect changes in cAMP levels following agonist stimulation of cells in the absence and presence of receptor antagonists.

In this study, we used the rat PC12 phaeochromocytoma cell-line which endogenously expresses adenosine A_{2A} and A_{2B} receptors. Here, we have utilised compounds selective for the A_{2A} receptor.

Materials & Methods

HitHunter® cAMP HS kit reagents (DiscoverX):

- Lysis buffer and antibody mixture
- ED reagent
- EA reagent and CL substrate mixture
- cAMP standard (0.25mM)

Other reagents and materials:

- Rat PC12 phaeochromocytoma cell-line (ATCC)
- Dulbecco's Phosphate Buffered Saline (PBS ; Cambrex)
- Microplate, low volume, white with clear bottom, tissue culture treated, sterile, 96-well (Corning)
- Agonist (CGS21680; Tocris)
- Antagonist (ZM241385; Tocris)
- IBMX (Sigma; optional)

A full description of the use of the HitHunter® cAMP assay is included with the kit. Low volume 96-well microplates were used.
To produce the standard curve, the cAMP standard provided in the kit was diluted 1 in 25 to prepare the highest working concentration, which was then used to prepare 1 in 3 serial dilutions in PBS giving a range of concentrations from 2.7×10^{-6}M to 4.6×10^{-11}M cAMP in a final assay volume of 55 µL. PBS alone was used as the control. PBS was also used, with PC12 cells, to measure the cAMP produced by constitutive receptor activity (basal activity).

Assay protocol (low volume 96-well plate):
PC12 cells were seeded 48 hours prior to the experiment at a density of 20,000 cells per well.

1. Add **cAMP standard** dilutions (15 µL) to empty wells
2. Remove media from the cells and resuspend them in **PBS** containing 500µM IBMX (10 µL)
3. Add **antagonist** to the cells (5 µL)
4. Incubate at 37°C for 15 min
5. Add **agonist** to the cells (5 µL)
6. Incubate at 37°C for 30 min
7. Add 10 µL of **Lysis buffer and antibody mixture** to each well
8. Incubate at room temperature for 60 min
9. Add 10 µL of **ED reagent** to each well
10. Incubate at room temperature for 60 min
11. Add 20 µL of **EA reagent and CL substrate mixture** to each well
12. Incubate at room temperature for at least 60 min

Rev. 09/2006
Keywords: **Agonist dose response, cAMP, GPCR, HitHunter, Receptor**

Chemiluminescence is then read on the BMG LABTECH microplate reader 4 hours after addition of the last reagent.

BMG LABTECH microplate reader settings:
Basic parameters for luminescence plate mode detection are listed below:

- Read mode: Plate
- Positioning delay: 0.2 sec
- No. of kinetic windows: 1
- No. of multichromatics: 1
- Emission filter: lens
- Gain: 3300-3600 (depending on reader)
- Measurement interval time: 1 sec

Results & Discussion

The HitHunter® cAMP HS assay from DiscoverX was used in 96-well format, and data from standard and agonist curves were obtained from the BMG LABTECH microplate reader in luminescence mode. Reagents were added according to the manufacturer's protocol, and chemiluminescence was read 4 hours after the addition of the last reagent. Data were evaluated using the MARS data analysis software and the software package GraphPad Prism.

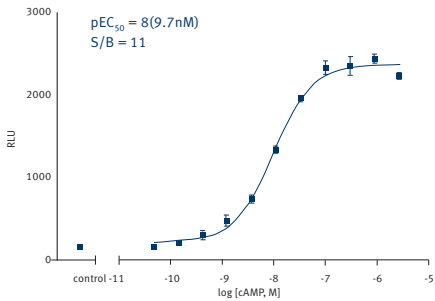


Fig. 2: cAMP standard curve for the HitHunter® cAMP HS assay (standards were measured in triplicate).

Fig. 2 illustrates the cAMP standard curve obtained using luminescence detection in a 96-well format. The curve shows a dose-dependent increase with a good signal-to-background noise (S/B) value of 11. Dose-response curves for the selective A_{2A} receptor agonist CGS21680 either alone or in the presence of the A_{2A} receptor selective antagonist ZM241385 were generated (Fig. 3).

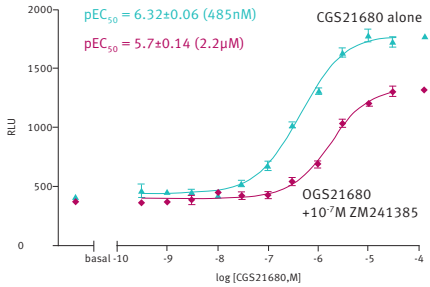


Fig. 3: Dose-response curves for CGS21680 in the presence or absence of ZM241385. pEC_{50} values were calculated, using GraphPad Prism software, from three individual experiments, each performed in triplicate.

A rightward shift of the agonist dose-response curve, and a decrease in the maximal response, was observed in the presence of 10^{-7}M ZM241385. This shows that ZM241385 non-competitively antagonised (by 4- to 5-fold) the agonist-induced increase in intracellular cAMP levels.

Conclusion

The HitHunter® cAMP HS assay is particularly suitable for detecting small changes in cAMP levels such as those seen in the rat PC12 phaeochromocytoma cell-line. Both agonist and antagonist data can be generated in conjunction with the BMG LABTECH microplate reader.

Promega's P450-Glo™ luminescent cytochrome P450 assay using a BMG LABTECH microplate reader

James Cali[1] and E.J. Dell[2]
[1] Promega Corporation, Madison, WI, USA [2] BMG LABTECH, Durham, NC, USA

- **Screen for cytochrome P450 inhibitors using Promega's P450-Glo™ assay on a BMG LABTECH microplate reader**
- **BMG LABTECH dedicated luminescence detection system gives a strong signal and minimizes background noise**
- **Rapid and reliable detection of cytochrome P450 activity**

Introduction

A large group of enzymes known as cytochromes P450 (CYP or P450) are responsible for the oxidative metabolism of many xenobiotic, hydrophobic chemicals, which include most therapeutic drugs. P450 metabolism can influence the clearance rate of drugs, their toxicity, and their interactions with other drugs. For drug discovery, researchers need to determine how new drug molecules are metabolized by P450 enzymes and how they may alter P450 activity. Some of these determinations can be achieved by using known P450 substrates as probes that change in a measurable way when they react with a P450. If the reactivity of the probe is altered in the presence of a drug, it can be concluded that the drug has an impact on P450 activity. As described herein, novel P450 inhibitors can be found if they reduce the reactivity of a P450 enzyme with a probe substrate.

BMG LABTECH microplate readers are flexible multifunctional microplate readers that can be equipped with a dedicated luminescence detection system. This system allows for the measurement of a strong luminescent signal, while keeping the background noise low.

Assay Design

P450-Glo™ Assays employ luminogenic P450 probe substrates that are derivatives of beetle luciferin, a substrate for luciferase enzymes. The derivatives are not substrates for luciferase but are converted by different P450 isoenzymes to luciferin (Figure 1), which in turn reacts with luciferase to produce a measurable amount of light that is directly proportional to the P450 activity.

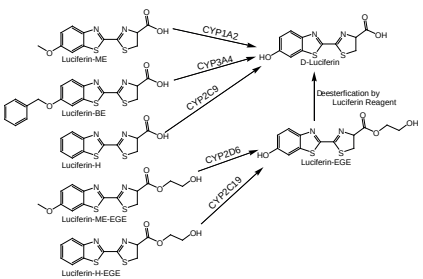


Fig. 1: P450-Glo™ luminogenic substrates converted by specific CYP isoenzymes to luciferin. (ME = methyl ether; BE = benzyl ether; EGE = ethylene glycol ether).

A luminogenic substrate is incubated with an active P450 preparation. The P450 activity is stopped and luciferin detected by adding Ultra-Glo™ Luciferase, a recombinant, stable luciferase in a proprietary buffer system that generates a "glow-type" luminescent signal (Figure 2). The half-life of the luminescent output is greater than two hours, allowing batch plate processing.

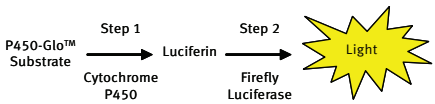


Fig. 2: P450-Glo™ luminescent assay steps.

Materials & Methods

- BMG LABTECH microplate reader
- Opaque, flat-bottom 96-well Costar® plates
- Promega's P450 Glo™ Screening System (contains baculovirus membrane preparation with human cytochrome P450 enzyme, luminogenic substrate (Fig. 1), NADPH regeneration solution, luciferin detection reagent, reaction buffer, and luciferin-free water) for: CYP1A2 (catalog # V9770), CYP2C9 (#V9790), CYP3A4 (#V9800), CYP2C19 (# V9880), and CYP2D6 (# V9890) D

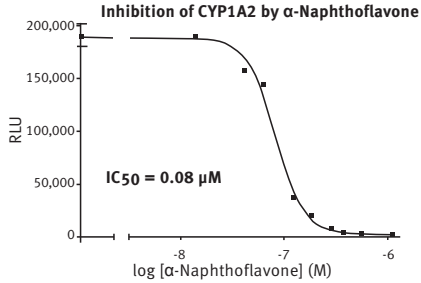
P450 assays were done with membrane preparations from insect cells co-expressed with P450 reductase or P450 reductase plus cytochrome b5. For background measurements membrane fractions with no P450 enzymes were used. P450 reactions were performed in opaque 96-well Costar® plates using a 50µl volume in KPO4 buffer (pH 7.4). P450 enzymes were incubated with their substrates for 10-30 minutes at 37°C. Reactions were initiated by the addition of an NADPH regenerating solution. P450 activity was stopped and luminescence was initiated by adding Luciferin Detection Reagent. Luminescence was read directly on the BMG LABTECH microplate reader in luminescence mode. The instrument's incubation chamber and onboard injectors can be easily used to automate the process. Each data point represents the average of three wells.

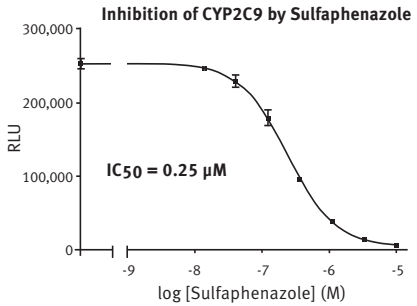
Results & Discussion

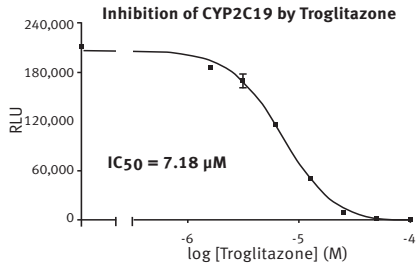
Using the BMG LABTECH reader, P450-Glo™ Assays can detect dose-dependent inhibition of recombinant P450s by known P450 inhibitors. Figure 3 shows dose related inhibition activity curves of the top five drug

Rev. 04/2007
Keywords: **CYP, Cytochrome P450, P450-glo, Luciferase, Luciferin**

metabolizers (CYP1A2, 2C9, 2C19, 2D6, and 3A4) and their respective inhibitors. The determined IC_{50} values are similar to values obtained with conventional probe substrates. This data demonstrates that a range of concentrations of a single compound can be screened to measure IC_{50} values, but it also implies that single concentrations of multiple test compounds can be screened against a chemical library.







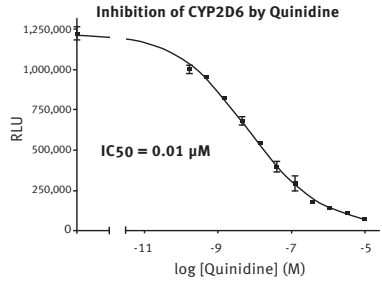


Fig. 3: P450-Glo™ IC_{50} calculations for CYP450 enzymes (CYP1A2, 2C9, 2C19, and 2D6) and their respective inhibitors (α-naphthoflavone, sulfaphenazole, troglitazone, and quinidine).

Conclusion

P450-Glo™ Assays provide a rapid, sensitive and highly reproducible approach to P450 screening. The systems are compatible with conventional P450 assay protocols in automated or manual multiwell plate formats. P450-Glo™ Luminescent Assays have several unique properties:

- Speed - The luminescent reaction is immediate and eliminates the need for time-consuming analyses
- Great Sensitivity - Low background levels and a large dynamic range result in sensitive assays that require less P450 enzyme
- Soluble Substrates - P450-Glo™ substrates are highly soluble in aqueous solutions
- Single readout - Multiple P450 isoforms with multiple substrates can be assayed at a single instrument setting
- Low false-positive rate - Proprietary stabilized firefly luciferase minimizes the number of false positives due to inhibition of luciferase by analytes when screening for P450 inhibitors

BMG LABTECH microplate readers provide the perfect platform to perform Promega's P450-Glo™ Assay. With a dedicated luminescence detection system the BMG LABTECH reader minimizes the background signal, thereby increasing the confidence in the data. Furthermore, with two optional onboard injectors and a standard 45°C incubation chamber, the BMG LABTECH reader can easily become fully automated to perform all of your P450 screening needs.

Evaluation of PARP inhibitors: performed on BMG LABTECH's FLUOstar® Omega

Rebecca Foster and Kyla Grimshaw
Hypoxium Ltd, Cambridge, UK

- **Cellular PARP assay and cell viability assay utilized to evaluate PARP inhibitors**
- **BMG LABTECH's FLUOstar® Omega allows versatility in choice of assay read out, allowing rapid detection of luminescence, fluorescence and absorbance**

Introduction

Poly ADP-ribosylation is a post-translational modification of proteins that plays a crucial role in regulating DNA repair. Poly (ADP-ribose) polymerase (PARP) transfers ADP-ribose to itself and other nuclear proteins such as histones. The substrate for that reaction is NAD+. PARP inhibition has been demonstrated to potentiate the cytotoxicity of anti-cancer drugs and ionising radiation. Therefore much effort has been put into the development of specific PARP inhibitors.

To evaluate such inhibitors, we have first used a specific PARP activity assay, to monitor their ability to inhibit endogenous PARP activity contained within a colon cancer cell line. This assay requires protein concentration determination (BCA assay - absorbance) followed by luminescent detection.

Secondly, we have used the AlamarBlue viability assay (fluorescent read-out) to evaluate the ability of a PARP inhibitor to potentiate the effects of the chemotherapeutic agent temozolomide to stimulate cell death in a colon cancer cell line. All measurements were performed using a FLUOstar Omega multidetection microplate reader from BMG LABTECH.

Materials & Methods

- Bicinchoninic Acid Protein Detection Kit from Sigma-Aldrich
- Universal Chemiluminescent PARP assay kit including white plates from Trevigen
- clear and black 96 well plates from Fisher
- Temozolomide from Sigma-Aldrich
- AlamarBlue from Invitrogen

Sample	Absorbance 560 nm
LoVo Control	0.767
LoVo 1 nM PARP Inhibitor	0.774
LoVo 3 nM PARP Inhibitor	0.791
LoVo 10 nM PARP Inhibitor	0.778
LoVo 30 nM PARP Inhibitor	0.762
LoVo 100 nM PARP Inhibitor	0.75
LoVo 300 nM PARP Inhibitor	0.736
Blank	0.075

Universal Chemiluminescent PARP assay

LoVo cells were treated with PARP inhibitor at a range of concentrations (1-300 nM) for 1h before cells were harvested and lysed in PARP Buffer.
A BCA protein assay was carried out in a 96 well microplate, following manufacturer's instructions adapted for a 96 well plate. The plate was read at 560 nm using the FLUOstar Omega.

A standard curve was generated for the BCA protein assay (Figure 1) and from this the protein concentration of the lysates were determined, and adjusted to 40 µg per sample.

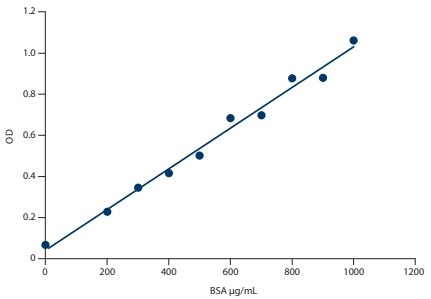


Fig. 1: BSA standard curve (linear regression fit performed using GraphPad Prism).

Lysates were then screened for PARP activity following manufacturer's instructions for determining PARP activity in cell and tissue extracts.
The assay measures the incorporation of biotinylated poly (ADPribose) onto histone proteins in a 96 well strip format. Detection is carried out using a Streptavidin-Horseradish Peroxidase (HRP) conjugate. Following addition of the HRP substrate, the resulting luminescent signal was read using the FLUOstar Omega equipped with luminescence optic.

AlamarBlue Viability Assay

LoVo cells were seeded in 96 well black plates at 5000 cells per well and allowed to adhere overnight, prior to addition of compound or vehicle control.
0- 300 µM temozolomide was added to cells, with and without 300 nM PARP inhibitor for 72 h. AlamarBlue 10 % (v/v) was then added to cells, and incubated for a further 6 h at 37°C. Live, metabolically active cells convert the AlamarBlue substrate to a fluorescent product, which was then detected using the FLUOstar Omega (Excitation 544 nm and Emisson 590 nm).

Rev. 04/2008
Keywords: **AlamarBlue, Cell proliferation, Cell viability, HRP, PARP**

Results & Discussion

Universal Chemiluminescent PARP assay

Following luminescence measurement, data was analysed using GraphPad Prism (Figure 2). The PARP inhibitor inhibits 50 % PARP activity at a concentration of 14 nM.

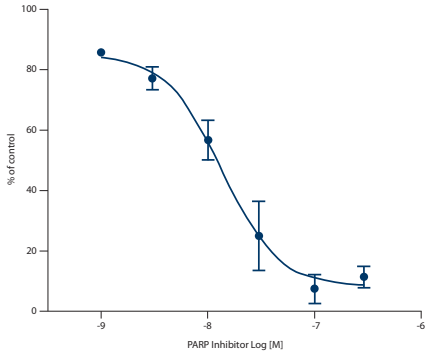


Fig. 2: Cellular PARP activity in LoVo cells treated with PARP inhibitor for 1h.

AlamarBlue Proliferation Assay

Following measurement of fluorescent product, data was analysed using GraphPad Prism (Figure 3). Temozolomide as a single agent leads to very little cell death, with an IC_{50} >300 µM.

However, addition of PARP inhibitor in combination with temozolomide, leads to a significant increase in cell death, with an IC_{50} of 60 µM. This represents >5-fold enhancement of cell death.

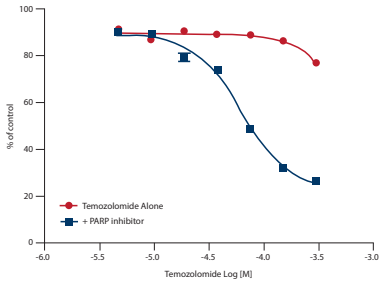


Fig. 3: Proliferation Assay performed in LoVo cells treated with Temozolomide alone, or in combination with 300 nM PARP inhibitor.

Note: PARP inhibitor alone does not stimulate cell death.

Conclusion

The ability of the FLUOstar Omega to measure absorbance, luminescence and fluorescence, facilitates simple, rapid measurement of all aspects of our evaluation, using a single machine.

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Omega Series

Dual luciferase assay to assess the replication of the hepatitis C virus subgenomic replicon

Sarah Gretton and Mark Harris
Institute of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT

- **DLR™ assay utilized to monitor early stage replication events of Hepatitis C virus**
- **Measurements performed on DLR™ certified BMG LABTECH microplate reader**
- **Potential inhibitors can be easily detected**

Introduction

Hepatitis C virus (HCV) is a global health problem affecting an estimated 170 million people worldwide. Chronic infection can lead to the development of cirrhosis resulting in end-stage liver failure or hepatocellular carcinoma. There is no vaccine for HCV and the current therapy is only effective in around 50 % of individuals. Therefore, improved understanding of the viral life cycle and systems for screening potential viral inhibitors are required.

The development of a subgenomic replicon system, encoding only the viral non-structural proteins required for RNA replication, allows the study of replication of the virus, without the release of infectious particles and the necessary containment facilities required for this work. Recently a subgenomic replicon has been generated from the efficiently replicating genotype 2a strain of HCV (JFH1), incorporating the firefly luciferase gene under the control of the HCV internal ribosome entry site element.

This has allowed monitoring of early stage replication events of HCV in the absence of any selective pressure, and furthermore investigation of the effects of compounds on these events.

This application note describes a method used for directly assessing the effects of potential inhibitors simultaneously on both the RNA replication of HCV, and overall translation within human hepatoma cells using a BMG LABTECH microplate reader. As an indicator for overall translation in the cell, a capped RNA transcript from the pRLTK plasmid was used. This plasmid contains a *Renilla* luciferase gene, preceded by a T7 promoter.

Materials & Methods

- BMG LABTECH microplate reader with injectors
- MEGAscript® T7 Kit, Ambion (Applied Biosystems)
- Cap Analog (m7G(5')ppp(5')G), Ambion (Applied Biosystems)
- Bio-rad Gene Pulser Xcell Eukaryotic System electroporator
- Gene Pulser/MicroPulser Cuvettes, 0.4 cm gap, Bio-rad

In addition, tissue culture consumables, pipette tips etc were used as needed from various manufacturers.

Dual luciferase Replication and Translation Assay

In vitro transcribed (IVT) replicon RNA was made from a linearised template of the JFH1 luciferase subgenomic replicon (SGR-JFH1-Luc) DNA using the MEGAscript® T7 Kit. In vitro transcription of linearised pRLTK plasmid DNA was as above but in the presence of a cap analogue. Four million PBS washed human hepatoma (Huh-7.5) cells were electroporated in 0.4 cm cuvettes with 5 µg of SGR-JFH1-Luc RNA and 5 µg of RTLK RNA simultaneously at 270 V and 950 µF.

Cells were then resuspended in standard cell media and seeded at a density of 1000 cells per well in 96 well plate. Cells were incubated as normal at 37°C. Cells were lysed directly in 96 well plates by carefully removing media and washing twice in PBS before adding 20 µL of 1 x Passive Lysis Buffer (Promega), and placing on a rocking platform (with gentle rocking to ensure even coverage of the cell monolayer) at room temperature for 15 minutes.

The 96 well plate containing the lysed cells was then either stored at -20°C (for up to a month) or read directly in the microplate reader using the following parameters:

Read mode: Luminescence, well mode kinetics
Positioning delay: 0.2 sec
Measurement start time: 0.0 sec
No of intervals: 48
Interval time: 0.50 sec
Emission Filter: empty/lens (depending on reader)
Recommended gain: 3800
Injection speed: 260 µL/sec
Injection start time: 1 and 13 sec

For data calculation, the relative luminescence units were averaged over two ranges:
Range 1 – Firefly luminescence (cycles 5-26 or 2 -12.5 secs)
Range 2 – *Renilla* luminescence (cycles 29-48 or 14-23.5 secs)

Results & Discussion

Figure 1 shows a typical signal curve of the experiment.

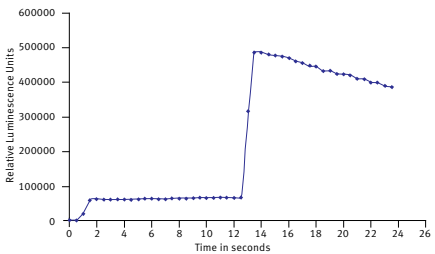

Fig. 1: Typical signal curve for the DLR assay. The substrate for the Firefly luciferase was injected in cycle 1, whereas the substrate for the *Renilla* enzyme was injected after 13 seconds.

Rev. 09/2008
Keywords: **DLR, Dual luciferase reporter, Firefly, Hepatitis C virus, Injectors, *Renilla***

Cells were harvested at 4, 8 and 24 hours post-electroporation, and analysed for Firefly and *Renilla* luciferase activity. Readings taken at 4 hours allowed establishment of input levels of RNA (Figs. 2 and 3).

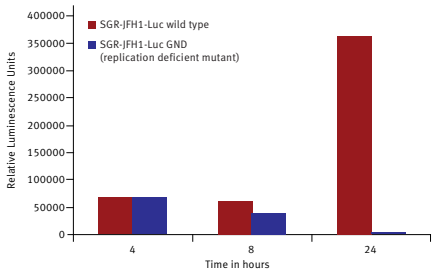

Fig. 2: Time course of SGR-JFH1-Luc wild type and replication deficient mutant (GND) luciferase levels over 24 hours.

For the first 4-8 hours translation of input SGR-JFH1-Luc RNA takes place, however, between 8 and 24 hours levels of luciferase increased 6 fold, indicating replication of the wild type replicon RNA, contrasting with luciferase levels of a replication deficient mutant (GND) which shows a decrease in luciferase activity after 8 hours (Fig. 2). Translation of RLTK RNA took place throughout the 24 hour time period but due to lack of replication and degradation of the RNA, the luciferase signal dropped over time (Fig. 3). A signal, however, was detectable at 24 hours allowing assessment of cellular translation.

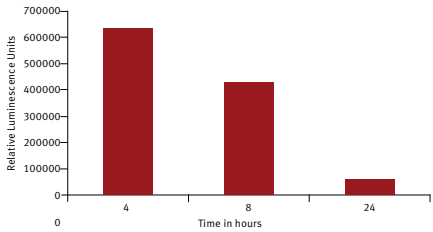

Fig. 3: Time course of RLTK RNA translation over 24 hours.

In order to assess the effects of various compounds on replication rather than translation, compounds were added 8 hours after electroporation and cells were incubated for a further 16 hours. Untreated cells were harvested at 4 hours (for normalisation of input RNA) and both treated and untreated cells were harvested at 24 hours to detect effects on replication (data not shown).

Conclusion

This assay allows evaluation of the effects of compounds on early stage replication events and on cellular translation. The 96 well plate format allows a number of compounds of varying concentrations to be tested simultaneously against cells derived from a single electroporation event.

The assay was successfully performed on the BMG LABTECH microplate reader. All BMG LABTECH microplate readers with luminescence detection and injectors are certified by Promega for the Dual Luciferase Reporter (DLR™) gene assay.

Multiplex analysis of inflammatory cytokines from primary human macrophages using a FLUOstar® Omega

Brenda E. Mutch, Kate Willetts, Emma Timms and Brian M J Foxwell
Kennedy Institute of Rheumatology, Hammersmith, London, UK

- **Protein concentration of different inflammatory cytokines determined**
- **Downscaling from 96-well to 384-well without loss of sensitivity**
- **FLUOstar® Omega used to measure ELISA and Luciferase assays**

Introduction

Rheumatoid Arthritis (RA) is a chronic autoimmune disease characterised by chronic inflammation and the accumulation of immune competent cells in joints. The cells that accumulate in joints include macrophages and T cells and these cells produce inflammatory mediators, in particular Tumour Necrosis Factor (TNF), that drive the inflammatory process. These cells also show an increased yet insufficient production of anti-inflammatory cytokines including IL-10.

Recent work from this laboratory and others has shown in both human and animal models of RA that there is a potential role for the TLR (Toll like receptor) family in driving cytokine production in RA.

TLRs are receptors that recognise 'Pathogen Associated Molecular Patterns (PAMPs)' such as lipopolysaccharide (LPS) or dsRNA that are associated with bacterial, viral or fungal infections. The limitation for screening potential new targets has always been the availability of sufficient tissue/primary human cells. By reducing the number of cells required in an assay it would be possible to test more targets and multiple outputs on each donor. In this application note we show different assays to monitor cytokine activity on BMG LABTECH's FLUOstar Omega. By reducing the number of cells we downscaled from 96 well to a 384 well format.

The FLUOstar Omega is a versatile, automated micro-plate reader that offers a range of detection modes: UV/Vis absorbance spectra, fluorescence intensity, time-resolved fluorescence, time-resolved FRET, luminescence (flash and glow) and AlphaScreen®.

Materials & Methods

- Capture and detection antibodies for human TNF-α, and IL-6 were purchased from BD Pharmingen
- Macrophage-colony stimulating factor (M-CSF) was purchased from Peprotech
- LPS was purchased from Alexa
- 96-well clear tissue culture plates
- 384-well clear tissue culture plates, Appleton Woods
- 96-well and 384-well Luciferase assay plates with solid strips
- 96-well and 384-well ELISA plates, Appleton Woods
- FLUOstar Omega, BMG LABTECH

Cells and Cell Culture

PBMCs (Peripheral Blood Mononuclear Cells) were prepared from buffy coat fractions of a unit of blood from a single donor using Ficoll-Hypaque (Nycomed). Monocytes were isolated by centrifugal elutriation (>85% purity), routinely collected and cultured in RPMI medium containing 10% heat inactivated foetal calf serum (FCS), as previously described. After that monocytes were differentiated in the presence of 100 ng/mL M-CSF (Peprotech) for 3 days. Non-adherent cells were washed off and the remaining adherent cells were removed using cell dissociation media (Sigma), counted and re-plated prior to assay. 96-well plates have 5x the growth area of 384-well plates so cell number and volume of media has been reduced by same ratio. Cells are stimulated by the addition of 10 ng/mL *E.coli* derived LPS (Alexa). All cell culture incubations were performed at 37°C in a humidified atmosphere containing 5% CO_2.

Generation of Adenoviral Vectors and Cell Infection

Recombinant, replication-deficient adenoviral constructs were prepared using the AdEasy system as previously described. MCSF derived macrophages were plated in a 96-well plate at $1x10^5$ cells/well, $2x10^4$ cells/well in 384-well plate and allowed to express adenoviral transgenes for at least 24 hours. Adenovirus used is the NFκB luciferase adenovirus (AdNFκB-luc) and contains four tandem copies of the κ enhancer element located upstream of the firefly luciferase gene. This adenovirus was provided by P.B. McCray Jr. (University of Iowa, Iowa City, IA) and is a modification of the pNFκB reporter vector (BD Clontech).

ELISA

Macrophages were plated at $1x10^5$ cells/well in a 96-well plate and at $2x10^4$ cells/well in a 384-well plate, incubated for 24 h at 37°C, and stimulated with 10 ng/mL LPS. Supernatants were harvested after 18 h and examined for concentrations of TNF-α and IL-6 following the manufacturers' instructions. Absorbance was read at 450 nm on a FLUOstar OMEGA and analysed using the MARS data analysis software. Results are presented as the mean concentration of triplicate cultures ± SD.

Luciferase Reporter Gene Assay

Cells were infected with a range of Moi (multiplicity of infection i.e. number of virus particles/cell) to generate a standard curve for GFP expression and luciferase units. After LPS stimulation, cells were washed once in PBS and lysed with 100 µL (96-well) or 50 µL (384-well) CAT lysis buffer. Cell lysates (50 µL 96-well or 25 µL 384-well) were transferred into a luminometer cuvette strip and (120 µL 96-well or 60 µL) luciferase assay buffer and 30 µL luciferin added as described previously. Luciferase activity was measured by detecting luminescence in relative luminescence units (RLU) using the FLUOstar Omega reader and MARS data analysis software.

Rev. 12/2008
Keywords: **Cytokines, ELISA, Inflammatory response, Luciferase reporter, Macrophages, TNFα**

Results & Discussion

After macrophage stimulation with LPS the concentration of TNF-α obtained in 96-well and 384-well format is comparable, as long as the ratio of cells and culture volume is kept constant to the growth area of the well (Fig 1).

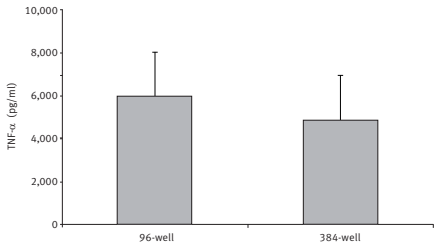

Fig. 1: Data from 3 donors comparing TNF-α concentration in supernatants from cells cultured in 96-well and 384-well plates.

IL-6 concentrations are also comparable between 96-well and 384-well plates (Fig 2).

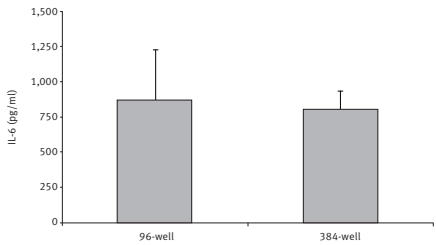

Fig. 2: Data from 3 donors comparing IL-6 concentration in supernatants from cells cultured in 96-well and 384-well plates.

ELISA measurements for quantifying further cytokines like IL-10 and IP-10 were also performed successfully in 384-well plates. The results were in accordance to the values obtained in the 96-well format (data not shown). Active cytokine NFκB was detected with the help of the luciferase assay (Figure 3).

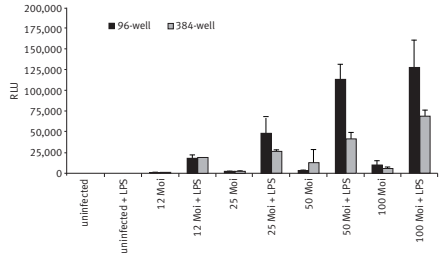

Fig. 3: Luciferase assay of human primary macrophages infected with NFκB Adenovirus in 96-well and 384-well format.

When using less than 25 Moi of virus sensitivity makes the assay unreliable.

The 96-well and 384-well results shown in Figure 4 are not comparable in their signal height as the 384 well plates were lysed in 2.5 fold more lysis buffer hence the relative luciferase units are approximately 2.5 fold lower than those obtained using 96 well plates. More lysis buffer was added to 384 well plates to ensure there was enough lysate to perform luciferase assay.

Conclusion

The FLUOstar Omega plate reader gives the potential to analyse multiple cytokines on the same assay plate when using the grouping feature. This pilot study shows that the human primary macrophages can be cultured in 384-well plates without any changes in morphology or cytokine expression.

Reducing the number of cells needed for each assay without altering the sensitivity will now open possibilities to do screening assays on primary cells to look for new targets in autoimmune diseases. Previously this has only been possible in cell lines that do not respond to TLR ligands in the same way as primary cells.

Successful Adenovirus infection of primary human macrophages in 384-well plates also allows reporter assays to be performed.

Promega's ADP-Glo™ kinase assay

Sarah Shultz[1], Franka Ganske[2] and E.J. Dell[2]
[1] Promega Corp. [2] BMG LABTECH

- **ADP-Glo™ Kinase Assay is a homogeneous luminescent assay to detect ADP**
- **ADP concentrations ranging from 1 mM to 0.01 µM can be detected**

Introduction

Kinases are a large and diverse group of enzymes that are involved in many cellular metabolic and regulatory processes. During the kinase reaction, substrates are phosphorylated while ATP is converted into ADP. Screening for active kinases or for kinase inhibitors is an important tool for the development of new drugs. To fulfil this need, Promega developed a kinase assay that is based on luminescence and the by-product ADP. The ADP-Glo™ Kinase assay is a universal, homogeneous, high-throughput screening method to measure kinase activity by quantifying the amount of ADP produced during the kinase reaction. This assay can be used to screen for any ADP-generating enzyme.
In this application note we will show results of the ADP-Glo Kinase assay obtained with multidetection microplate readers from BMG LABTECH.

Assay Principle

The principle of the assay is presented in figure 1.

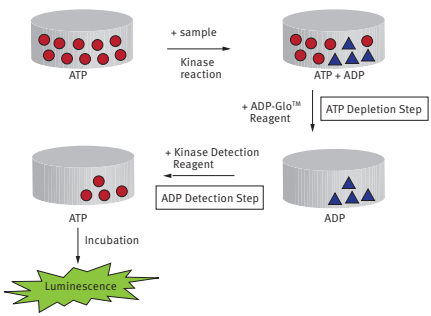

Fig. 1: Principle of the ADP-Glo™ Kinase Assay.

The assay consists of two steps. After the enzymatic reaction is finished, ATP and ADP are present in the well. The first step is to add the ADP-Glo™ Reagent resulting in the elimination of the remaining ATP. The second step is to convert the remaining ADP into ATP by adding Kinase Detection Reagent. This reagent also contains everything needed to measure the newly generated ATP with the help of a luciferase/luciferin reaction. The luminescence measured after incubation is proportional to the ADP concentration generated during the enzymatic reaction.

Materials & Methods

- White 384-well small volume plates from Greiner
- ADP-Glo™ Kinase Assay from Promega, including ATP and ADP
- BMG LABTECH microplate reader

To mimic an enzymatic reaction, an ADP/ATP standard curve was prepared from the nucleotide stock solutions that were supplied with the kit. The assay allows ADP measurements from 1 mM ADP to 0.01 µM ADP. Four different ADP/ATP standard curves were prepared: 1 mM, 100 µM, 10 µM and 1 µM. As an example, the dilution table for a 1 mM ATP/ADP standard dilution is given below. All other standard curves were diluted accordingly.

mM	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
ADP	1	0.8	0.6	0.4	0.2	0.1	0,05	0.04	0.03	0.02	0.01	0
ATP	0	0.2	0.4	0.6	0.8	0.9	0.95	0.96	0.97	0.98	0.99	1

Five µL of standard dilution was mixed with 5 µL of ADP-Glo™ reagent followed by a 40 min incubation at room temperature. After that 10 µL of Kinase Detection Reagent was then injected with onboard injectors. The signal was measured each minute for 40 minutes. Alternatively it is possible to measure endpoint luminescence after a 40-60 min incubation at room temperature.

Instrument settings for a kinetic reaction

Detection Mode:	Luminescence, plate mode
Measurement time:	1 sec
Cycle Time:	60 sec
Cycles:	40
Optics:	dedicated luminescence optic
Gain:	Optimized for the ADP concentration

Results & Discussion

Fig. 2 shows a signal curve for a 1 mM ADP/ATP standard dilution.

Rev. 12/2009
Keywords: **ADP, ADP-Glo, Injectors, Kinase, Luciferase, Luciferin**

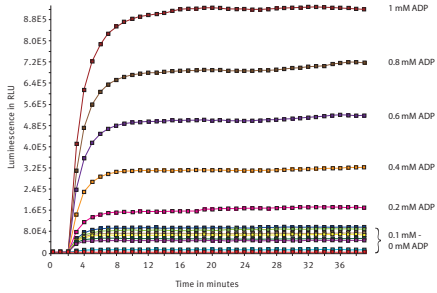

Fig. 2: Signal curves for several dilutions of ADP/ATP standard measured on the PHERAstar® *FS*. The gain was set to 2400.

The signal curves show that already after 20 min the signal is very stable. The 12 standards show different signal heights according to their ADP concentration. The last 10 data points of the signal curves were averaged and taken as a base for a linear regression fit.

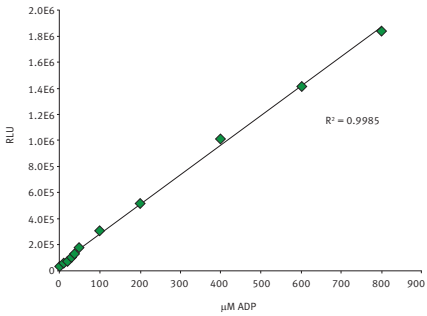

Fig. 3: 1 mM ADP/ATP standard curve.

Such a fit for a 1 mM nucleotide standard curve can be seen in Fig. 3. The same gain setting was used to create data for a 1 µM standard curve (Fig. 4)

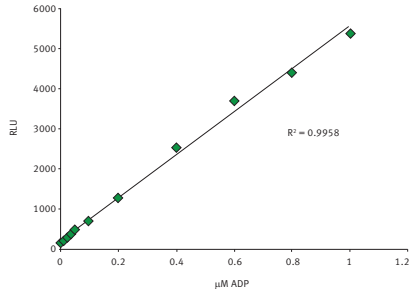

Fig. 4: 1 µM ADP/ATP standard curve.

It is possible to measure standard curves from 1 mM to 1 µM ADP using the same gain settings indicating a high assay window.

Conclusion

The data in this application note proves that BMG LABTECH microplate readers can successfully perform the ADP-Glo™ Kinase Assay from Promega in 384-well format using only 20 µl. Equipped with on board injectors, it is possible to choose to follow the reaction online using the kinetic mode or as an endpoint measurement.

BlueScreen HC™ - a luminescence based, high-throughput, in vitro genotoxicity assay

Andrew Knight, Chris Hughes and Matthew Tate
Gentronix Limited, Manchester, UK

- **Protocol for the rapid and accurate detection of genotoxic liability in various test articles**
- **Test articles include pharmaceuticals, industrial chemicals & personal care products**
- **Genotoxicity & Cytotoxicity measured simultaneously using flash luminescence, absorbance and fluorescence**

Introduction

The BlueScreen HC™ genotoxicity assay from Gentronix Ltd. uses a human-derived, p53-competent, TK6 cell line to host a luminescence-based reporter system that exploits the proper regulation of the GADD45a gene. The assay generates positive results for direct-acting mutagens and clastogens, as well compounds that act indirectly such as aneugens, and topoisomerase and polymerase inhibitors, which interfere with the processes of DNA replication, maintenance, repair and segregation. Importantly, correct negative results are produced for non-carcinogens, many of which give misleading positive results in other *in vitro* genotoxicity tests. In the BlueScreen HC S9 assay a metabolic activation protocol using rodent liver extract (S9) extends the range of compounds detected to include genotoxic metabolites. S9 contains cytochrome P450s and other enzymes that catalyse the detoxification of xenobiotic substances.

The BlueScreen HC assay utilizes a luciferase from the marine copepod *Gaussia princeps* to generate a luminescent output. Exposure to a genotoxic chemical causes increased expression of GADD45a and thus a dose dependent increase in the production of luciferase from the GADD45a reporter, which is naturally exported from the cell. The amount of luciferase produced by the cells is assessed by injection of a solution of the substrate, coelenterazine, resulting in a short-lived flash luminescence. In addition, reduced cell proliferation, a measure of cytotoxicity, is assessed either by changes in optical absorbance in the BlueScreen HC assay, or in fluorescence from a DNA stain in the BlueScreen HC S9 assay.

Materials & Methods

- BlueScreen HC and BlueScreen HC S9 reagent kits and cell line
- BlueScreen HC – Black, clear flat-bottomed, sterile, 96-well microplates from Thermo Scientific Matrix.
- BlueScreen HC S9 – Black, solid round-bottomed, sterile, 96-well microplates from Greiner Bio-One.

Preparation of the Assay Microplates

The genetically modified reporter cells are maintained in an RPMI based culture medium and grown to a density between 0.5 and 1.2 x 10^6 cells/mL in preparation for the assay. 4 test articles (i.e. pure chemicals, compound mixtures or product formulations) are tested over 8 serial (2-fold) dilutions, together with the appropriate genotoxic positive control, in one 96-well microplate. After arraying the test compounds in duplicate, an equal volume (75 µL) of growing cells at a density of 2 x 10^6 cells/mL is added to each well in columns 1 to 11. Microplates are covered with a breathable membrane and incubated at 37°C, 5% CO_2 and 95% humidity without shaking. For studies without S9 metabolic activation the microplates are incubated for 48 hrs. For studies incorporating S9 metabolic activation, S9 is added to each well of the microplate containing cells at a final concentration of 1% v/v S9. After 3 hrs the cells are washed and then re-suspended in fresh recovery media for a further 45 hrs.

BlueScreen HC and BlueScreen HC S9 parameters

Microplates are shaken to re-suspend the cells before measurement. For the BlueScreen HC assay (without S9) the microplate wells are first assessed using absorbance measurements to determine cell density, then using flash luminescence to quantify luciferase expression. For the BlueScreen HC S9 assay, luciferase expression is quantified first, followed by cell lysis and assessment of cell density using thiazole orange which is fluorescent when bound to DNA. Reduced cell density compared to untreated cells provides a measure of cytotoxicity. Luminescence intensity, corrected for cell density, is proportional to the expression of GADD45a and thus the genotoxicity of the test article. Table 1 summarizes the differences between protocols in the absence and presence of S9.

Instrument settings:

- Absorbance: 620 nm using 20 flashes per well.
- Fluorescence: Excitation 485 nm, emission 520 nm, optimal gain, top reading and 10 flashes per well.
- Luminescence: "well mode", injecting, shaking and reading each well sequentially. 50 µL of a 2.5 µM solution of coelenterazine was injected into the well using an in-built reagent injector at a speed of 260 µL/s. The microplate was then shaken for 1 s (500 rpm, orbital) immediately after which luminescence was recorded with an integration time of 5 s.

Table 1: BlueScreen HC assay protocols, run either in the absence or presence of S9 fraction.

	BlueScreen HC	*BlueScreen HC S9 with metabolic activation*
Metabolic activation	-	1% v/v S9 Fraction
Incubation Time	Exposure - 48 hours	Exposure - 3 hours / Recovery - 45 hours
Positive Control Compound	4-Nitroquinoline oxide (0.5 and 0.125 µg/mL)	Cyclophosphamide (25 and 5 µg/mL)
Luciferase Assay	Flash Luminescence	Flash Luminescence
Relative Cell Density Assessment	Optical Absorbance	DNA Stain Fluorescence
Microplate	Black, clear flat-bottomed, sterile, 96-well	Black, solid round-bottomed, sterile, 96-well

Rev. 08/2011
Keywords: **BlueScreen, Cell density, Cytotoxicity, Genotoxicity, Luciferase reporter, Microbial growth**

Data Handling

Absorbance, fluorescence and luminescence readings are exported directly into an Excel-based software template supplied by Gentronix. The software gives automated decisions (i.e. positive, negative), quantitative results (i.e. lowest effective concentration) and clear graphical dose response data for both genotoxicity and related cytotoxicity endpoints. Results for wells containing the test article are scaled relative to the vehicle (diluent) treated controls. Positive results are defined according to statistically derived threshold values for an increase in luminescence induction for genotoxicity and a decrease in relative cell density for cytotoxicity.

Results & Discussion

A range of compounds were tested with varying toxic properties.

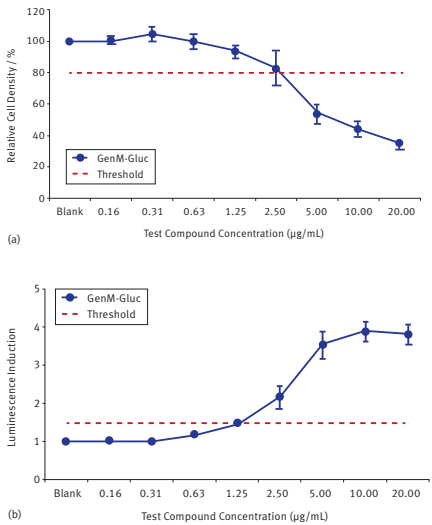

Fig. 1: BlueScreen HC S9 assay positive cytotoxicity (a) and genotoxicity (b) results for 20 µg ml benzo[a]pyrene. Error bars show +/-1 standard deviation based on 4 replicate analyses on separate microplates.

BlueScreen HC results are given in Table 2 and include 2 genotoxins (methyl methanesulfonate and 4-nitroquinoline oxide) and 1 cytotoxic non-genotoxin (2,4-dichlorophenol). BlueScreen HC S9 results are given in Figure 1 and Table 3 and include 3 pro-genotoxic chemicals that form genotoxic metabolites in the presence of S9.

Table 2: Cytotoxicity and genotoxicity results for 4 test articles, screened in the BlueScreen HC assay. LEC = Lowest effective concentration.

Test Article	Concentration (µg/mL)	Cytotoxicity	LEC (µg/mL)	Genotoxicity	LEC (µg/mL)
Methyl Methanesulfonate	50	POSITIVE	12.5	POSITIVE	6.25
4-Nitroquinoline Oxide	1	POSITIVE	0.25	POSITIVE	0.13
2,4-Dichlorophenol	324	POSITIVE	40.5	NEGATIVE	-
Vehicle Control	-	NEGATIVE	-	NEGATIVE	-

Table 3: Cytotoxicity and genotoxicity results for 4 test articles, screened in the BlueScreen HC S9 assay, incorporating exogenous metabolic activation for the detection of genotoxic metabolites. LEC = Lowest effective concentration.

Test Article	Concentration (µg/mL)	Cytotoxicity	LEC (µg/mL)	Genotoxicity	LEC (µg/mL)
Aflatoxin	1.25	POSITIVE	0.08	POSITIVE	0.04
6-Aminochrysene	40	POSITIVE	5	POSITIVE	2.5
Benzo[a]pyrene	20	POSITIVE	5	POSITIVE	2.5
Vehicle Control	-	NEGATIVE	-	NEGATIVE	-

LECs obtained for these well-characterised compounds were the same or within 1 serial dilution of historic controls and highly consistent between replicates.

Conclusion

The BlueScreen HC and BlueScreen HC S9 assays are shown here to be highly compatible with the BMG LABTECH microplate reader making use of the 3 principal detection modes and the reagent injection system. Using the instrument the BlueScreen HC assay has been shown to be fast, accurate and reproducible in the detection and quantification of genotoxic liability of a broad range of chemicals with differing potencies and modes of action.

Nano high-throughput screening (nHTS) platform - miniaturization of cell-based GPCR and kinase assays

E.J. Dell[1], Sunitha Sastry[2], Bonnie Edwards[3] and Jing Wang[3]
[1]BMG LABTECH [2]DiscoverX [3]Labcyte Inc.

- **Cell-based, nanoHTS platform can screen as little as 250 cells/well in a volume less than one microliter**
- **PathHunter® GPCR and Kinase cell-lines were dispensed using the Echo 555 liquid dispenser**
- **PHERAstar® *FS* was used for detecting the signal in 1536- and 3456-well microplates**

Introduction

With the development of more sensitive technologies, further miniaturization of cell-based assays for high-throughput screening is now very feasible. Performing cell-based assays in sub microliter volumes, translates into very low compound requirement and fewer cells used. In addition to being more economical, more experiments can be done in the same amount of time and at the same overall price.

Combining technologies from three companies, an nHTS platform was developed to screen GPCRs (shown here) and kinases (data not shown here) in a cell-based assay format in 1536 and 3456-well plates. Technologies used include DiscoverX's PathHunter® GPCR beta-Arrestin and PathHunter® Cell-based Kinase assay platforms; Labcyte's Echo® liquid handling system to transfer cells and compounds; and BMG LABTECH's PHERAstar *FS* microplate reader for low volume detection.

Assay Principle

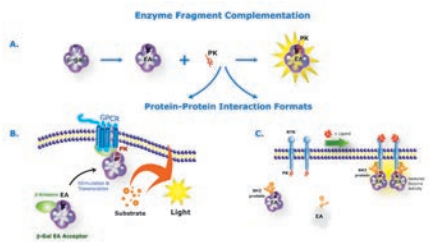

Fig. 1: PathHunter® assay principle.

The PathHunter® technology from DiscoverX is an adaptation of EFC that provides a novel cell-based assay format for detecting protein-protein interaction. In this approach, ProLink™ is appended to GPCR (1B) or RTK or cytokine receptor (1C), while EA is recombinantly expressed as a fusion protein with β-Arrestin (Fig 1B) or SH2 phosphotyrosine domain (Fig. 1C). Activation of the receptor upon ligand binding allows interaction of GPCR-β-Arrestin or RTK-SH2 protein resulting in a measurable chemiluminescent signal.

Materials & Methods

- PHERAstar *FS* from BMG LABTECH
- Echo® 555 liquid handler from Labcyte
- PathHunter® cell lines and assay reagents from DiscovrX
- 1536- and 3456-well microplates from Aurora

Cell Handling

PathHunter® Cells were diluted in Hank's Buffered SalineSolution. 40 µL were added to Echo® qualified 384-well polypropylene microplates (P-05525). PathHunter® Cells were transferred to an Aurora 1536-well or 3456-well microplate using an Echo® 555 liquid handler with a 384PP_AQ_SP calibration. Cell volumes were varied as part of the testing plan. Compounds such as pergolide, ProLactin or β-NGF were diluted in DMSO and added to an Echo® qualified 384-well polypropylene microplate and transferred at 30 nL using the 384PP_DMSO calibration. Incubation as per β-arrestin and Cell-based Kinase Assay recommendations.

Addition of Detection Reagents

PathHunter® Flash Detection reagent was transferred from an Echo® qualified 384-well microplate and transferred (at a 1:1 ratio of cell volume: PathHunter® Flash Detection reagent) with the Echo® 555 liquid handler with the 384PP_AQ_SP calibration. Incubate for 30 mns at RT.

Detection

Both 1536- and 3456-well microplates were measured on the PHERAstar *FS* using a measurement time of 0.1 sec/well at a gain of 3600 with a luminescence optic module. An automatic z-height adjustment was performed on the well with the highest signal before reading the plate. The 0.1 mm adjustable z-height allows the PHERAstar *FS* to obtain the highest possible signal-to-noise ratios in all cell-based assays.

Results & Discussion

Adherent Format

PathHunter® U2OS cells expressing Trk A and PRLR-JAK1 at 1000 cells/well were seeded in a white TC coated AURORA 1536 plates and stimulated with known agonists β-NGF and prolactin. These plates were incubated for 3 hrs at 22°C. A robust assay response is measured with 6 µL total volume (Figure 2).

Rev. 12/2012
Keywords: **3456-well, Arrestin, Kinase, NanoHTS, PathHunter**

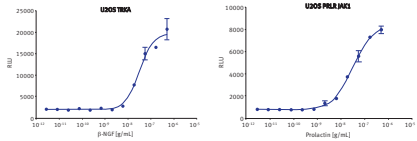

Fig. 2: PathHunter® U2OS cell receptor tyrosine kinase assay in a 1536-well adherent format stimulate with prolactin (left graph) and β-NGF (right graph) using 1000 cells/well.

PathHunter® CHO-K1 cells expressing Long isoform of Dopamine receptor D2 were seeded in a white TC coated AURORA 1536 plates at 1000 cells/well and incubated overnight for cells to adhere. Cells were stimulated with Pergolide for 90 minutes at 37°C (figure 3).

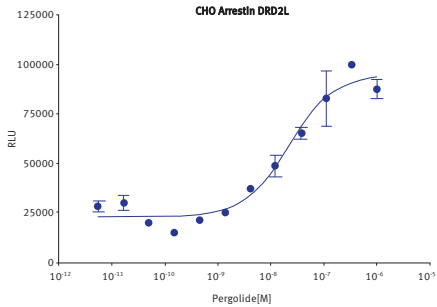

Fig. 3: Adherent CHO cells with PathHunter® β-Arrestin Assay measuring the Dopamine 2L receptor (DRD2L) in a 1536-well using 1000 cells/well.

These results indicate that the PathHunter® arrestin (and kinase) assay can be successfully adapted to detection in 1536 well microtiter plates using adherent cells.

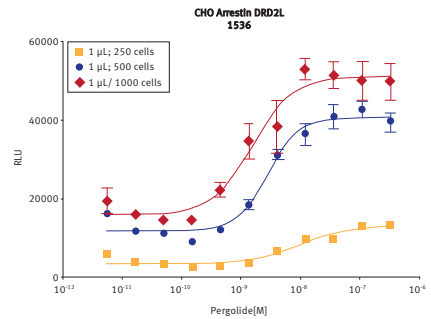

Fig. 4: Cell-seeding assay. PathHunter® DRD2L cells from 250 to 1000 cells/well in suspension were added to 1536 well plates in a 1 µL volume and stimulated with 5-10 nL of 100X pergolide (in DMSO) added to cells via the Echo® Liquid handler.

Different suspensions of CHO cells are shown in figure 4, the DRD2L 1536 suspension protocol produced dose curves similar to the adherent cell experiment. For optimal performance it is recommended that cell seeding volume using the Echo® instrument not exceed 1µL.

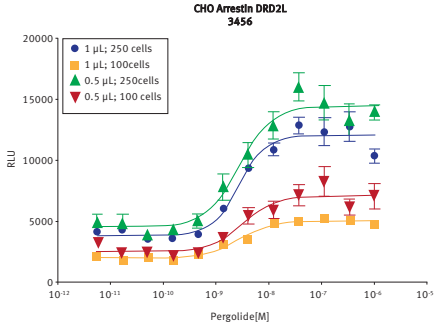

Fig. 5: 3456-well Protocol Optimization. PathHunter® DRD2L cells 100 or 250 cells/well (in suspension) were added to the AURORA 3456 well plates in 0.5 or 1 µL volumes via the Echo® instrument. Compound (5nl of 100X in DMSO) was added to the cells plated in 0.5 µl (10 nl for the cells plated in 1µl) via the Echo® instrument.

The protocol for cell seeding using the Echo® 555 was applied to cell dispensing into 3456 well plates, figure 5. The performance of different cell densities was tested to determine the optimal cell number in the new plate format. PathHunter® DRD2L cells 100 or 250 cells/well (in suspension) were added to the AURORA 3456 well plates in 0.5 or 1 µL volumes via the Echo® instrument. Compound (5nl of 100X in DMSO) was added to the cells plated in 0.5 µl (10 nl for the cells plated in 1µl) via the Echo® instrument.

Conclusion

Miniaturization with highly evolved automation technologies affords the benefit of faster screens at much lower costs. Here we have demonstrated the application of the DiscoverX's PathHunter GPCR and cell-based kinase assays in 3456 and 1536 platforms using Labcyte's ECHO Liquid handler and BMG LABTECH's PHERAstar *FS*. We describe an nHTS cell-based screening platform that can measure as little at 250 cells per well using less than 1 microliter.

PHERAstar® *FSX*
PHERAstar® *FS*

Simultaneous Dual Emission detection of luciferase reporter assays

Megan Dobbs[1], Douglas Hughes[1], Janaki Narahari[1], Jae Choi[1], Georgyi Los[1], Brian Webb[1], Eric Matthews[2], and Carl Peters[2]
[1]Thermo Fisher Scientific; [2]BMG LABTECH

- **Performing dual-luciferase assays using the microplate reader from BMG LABTECH**
- **Spectral resolution allows detection of two luciferase activities in one sample**
- **Simultaneous dual-emission detection permits one read assessment**

Introduction

Luciferase-based reporter assays employ insertion of a genetic regulatory element upstream of the luciferase gene. The result is an excellent tool for monitoring gene expression in cells due to wide dynamic range and sensitive detection. The luminescence resulting from expression of the transfected luciferase reporter gene is measured to quantify the activity of the cis-acting or trans-acting components of the biological pathway. In dual luciferase assays the expression values of one luciferase is usually related to the studied subject whereas the other luciferase reporter is considered as an indicator for transfection efficiency and cell viability.

Assay Principle

The Thermo Scientific™ Pierce™ Luciferase Dual-Spectral Assays provide a simple one-step detection protocol based on the use of pairs of luciferase enzymes that are spectrally resolved. Therefore their activities can be measured with high sensitivity in the same samples (Figure 1) without the usual quenching step needed to differentiate luciferase signals. Three dual assay systems in which red firefly luciferase is paired with green *Renilla*, *Gaussia* or *Cypridina* luciferase are available.

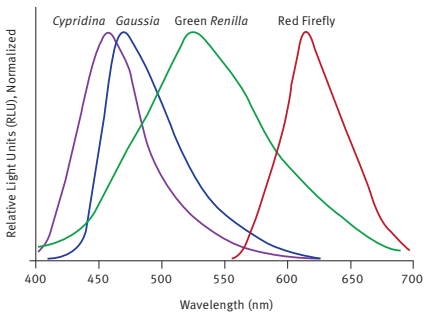

Fig. 1: Spectral emission profiles of luciferases used in Thermo Scientific™ Pierce ™Dual-Spectral Assay Kits. The emission of red firefly (λmax = 613nm) allows resolution from Green *Renilla* Luc (λmax = 535nm), *Gaussia* Luc (λmax = 470nm) and *Cypridina* Luc (λmax = 463nm).

Detecting activity from two spectrally resolved luciferases is a task for which the BMG LABTECH multidetection microplate reader is ideally suited. Light from both reporters can be measured either using the simultaneous dual-emission option which uses two photomultiplier tubes (PMT), or it can be measured sequentially, one emission after the other. Here we demonstrate the sensitivity of the Pierce™ Luciferase Dual-Spectral Reporter Assays, over at least a 100,000-fold luciferase concentration range after normalization to a control reporter, typically red firefly luciferase.

Materials & Methods

- HEK293 stable cell lines expressing *Cypridina*, *Gaussia*, Green *Renilla* and Red Firefly luciferase under the control of CMV promoter
- Pierce™ Luciferase Cell Lysis Buffer
- Pierce™ *Cypridina*-Firefly Luciferase Dual-Spectral Assay Kit
- Pierce™ *Gaussia*-Firefly Luciferase Dual-Spectral Assay Kit
- Pierce™ *Renilla*-Firefly Luciferase Dual-Spectral Assay Kit
- White, 96-well f-bottom plates (Thermo Scientific)
- BMG LABTECH microplate reader

HEK293 cell lines stably expressing *Cypridina*, *Gaussia*, green *Renilla* and red firefly luciferases were lysed with Pierce™ Luciferase Cell Lysis Buffer. 1:10 dilutions were prepared in lysis buffer. Analysis of replicates of each serial dilution (10 µL/well) was performed. Cell lysate containing a different second reporter (10 µL/well) was added as a control. For serial dilutions of cell lysate containing *Cypridina*, *Gaussia*, or green *Renilla* luciferase, the second control reporter was red firefly. For serial dilutions of cell lysate containing red firefly, the second control reporter was *Cypridina* luciferase. The appropriate Pierce™ Luciferase Dual Assay Working Solution was prepared for each assay, and 50 µL/well was injected into each well for a final volume of 70 mL/ well. Luminescence was read through appropriate filters with a 1 second integration time. A more detailed protocol can be found online. An overview of the filter settings is given in table 1.

Table 1: Filter settings for each dual-spectral luciferase assay.

Assay	Luciferase	Filters	Gain
Cypridina-Firefly	*Cypridina*	475 +/- 30	2500
	Red Firefly	610 long pass	2000
Gaussia-Firefly	*Gaussia*	475 +/- 30	2500
	Red Firefly	610 long pass	2000
Renilla-Firefly	*Renilla*	515 +/- 30	2000
	Red Firefly	670 +/- 10	3000

Rev. 03/2013
Keywords: **Cell viability, *Cypridina*, DLR, Dual luciferase reporter, *Gaussia*, Simultaneous Dual Emission SDE, Transfection efficiency**

Results & Discussion

Measuring the separate signals in each of the three pairs of luciferase reporter enzymes in the Pierce™ Luciferase Dual-Spectral Assay Kits was easy, using the appropriate filters (Figures 2, 3 and 4).

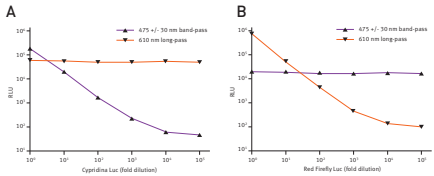

Fig. 2: Cypridina-Firefly Dual-Spectral Luciferase Assay on the POLARstar Omega using the instrument's dual-emission optics. A. Serial dilutions of HEK293 cell lysate expressing *Cypridina* Luc (n = 6). HEK293 lysate expressing Red Firefly Luc as a control. **B.** Serial dilutions of HEK293 cell lysate expressing red firefly luciferase (n = 6). HEK293 lysate from expressing *Cypridina* Luc as a mock "control".

By selecting filters for the luciferases in each pair that eliminate or greatly reduce interference between them, conditions for each dual-color assay system can be found that provide accurate measurement over 100,000-fold concentration range for each reporter (Figures 2 and 3).

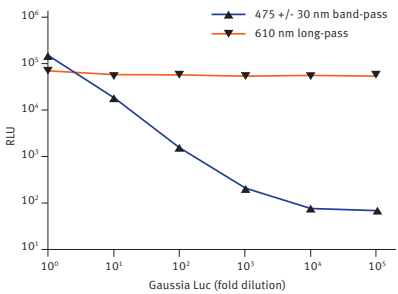

Fig. 3: Gaussia-Firefly Dual-Spectral Luciferase Assay. Serial dilutions of HEK293 cell lysate from a stable cell line expressing *Gaussia* Luc (n = 6). HEK293 cell lysate from a stable cell line expressing Red Firefly Luc was also added as a control.

Filter selection is critical for eliminating interference when spectral overlap is present between reporters (Figure 1). Thus for the *Renilla*-firefly assay, we used a suboptimal red-shifted 670 +/- 10nm bandpass filter for detection of red firefly, which minimizes interference from green *Renilla*. As a result, interference was only observed when the output from green *Renilla* was significantly greater than that from red firefly (Figure 4). To separate spectral data in this region a corrective calculation is needed (see Pierce™ Dual-Spectral Calculator).

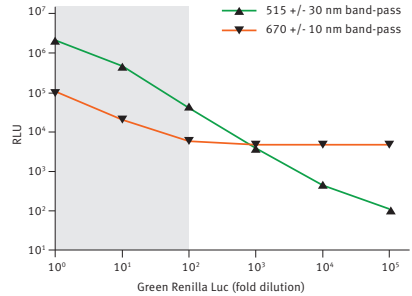

Fig. 4: Renilla-Firefly Dual-Spectral Assay. Serial dilutions of HEK293 cell lysate from a stable cell line expressing Green *Renilla* Luc (n = 6). HEK293 cell lysate from a stable cell line expressing red firefly luciferase was also added as a control. The shaded region indicates significant bleed-through of green *Renilla* luminescence into the red filter.

Conclusion

These experiments confirm the BMG LABTECH´s microplate reader's ability to measure the activity of two luciferases that are spectrally resolved. The Thermo Scientific™ Pierce™ Luciferase Dual-Spectral Reporter Assays are sensitive over at least a 100,000-fold dilution range of each luciferase when measured using appropriate filters. Defining the limit of detection in a dual-luciferase assay is difficult and will depend upon luciferase concentration as will the dynamic range.

Screening for histone deacetylase (HDAC) active compounds

Franka Maurer[1], Sheraz Gul[2] and Gesa Witt[2]
[1] BMG LABTECH [2] European ScreeningPort GmbH

- **Active compound screening in 384-well format plates using a chemiluminescent assay**
- **Proof-of-Concept Screen and Counter Screen data fast and reliably obtained on the PHERAstar® *FS* using the screening facility at the European ScreeningPort**

Introduction

Histones are small basic proteins. DNA in a stacked configuration is bound around histones to build chromatin prior the replication procedure. After translation, histone side chains are often modified. This includes acetylation and deacetylation as well as methylation, ADP-ribosylation, and phosphorylation. Histone deacetylation is carried out by enzymes that build a complex with histones. These complexes target specific promoters to repress transcription through deacetylation of histones in specific nucleosomes.
HDAC inhibitors (HDACi) have been used to treat neurological symptoms, as well as cancers, parasitic and inflammatory disease. In order to find compounds that are potential inhibitors of HDAC activity (Class I and II), a chemiluminescent Proof-of-Concept Screen was performed on the PHERAstar *FS* microplate reader from BMG LABTECH.

Assay Principle

The HDAC-Glo™ I/II assay from Promega measures the activity of HDAC class I and II (Fig. 1). An acetylated peptide is offered as an HDAC substrate. After deacetylation, a protease is added that cleaves the deacetylated substrate and releases aminoluciferin that is immediately consumed by a luciferase. The resulting luminescent signal is proportional to the HDAC activity.

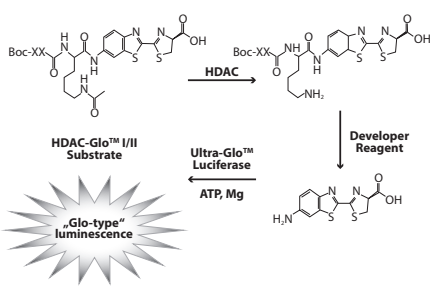

Fig. 1: Assay Principle for the HDAC-Glo I/II assay from Promega.

Materials & Methods

- HDAC-Glo I/II from Promega
- white 384 well plates, small volume, non-binding
- Echo liquid handling system from Labcyte
- PHERAstar *FS* microplate reader from BMG LABTECH

Dose response curve of a standard inhibitor
Dissolve the inhibitor in DMSO and prepare a dilution series in DMSO in a Labcyte plate. Transfer the inhibitor to an assay plate using the Labcyte Echo. Dilute enzyme to the desired concentration (2x final concentration) in assay buffer. Dilute developer solution 1:1000 in substrate solution. Add 5 µl of enzyme to each well of the plate and seal. Centrifuge plate 1 min. Add 5 µl of developer reagent to each well. Seal assay plate. Centrifuge the plate 1 min. Shake plate on a plate shaker for 30 seconds. Incubate plate at room temperature for 10 min. Detect the luminescence signal on the PHERAstar *FS*.

Proof-of-Concept Screen and Counter Screen
50 nl of compounds from the library are added with the Labcyte Echo liquid handling system. Add 5 µl of enzyme, seal and centrifuge. Then add 5 µl of reagent, seal, centrifuge, and shake the plate as described above. After an incubation of 10 min the plate is read in the PHERAstar *FS*. To identify false positives, a Counter Screen was performed using a control substrate which contains deacetylated substrate. This control substrate is diluted 1:10,000 in assay buffer. The to be tested compounds are transferred to an assay plate using the Labcyte Echo and 5 µl of control substrate added to each well. Centrifuge plate for 1 min and shake for 30 seconds. No enzyme should be added. Add 5 µl of reagent, seal, centrifuge, and shake the plate as described above. After an incubation of 10 min the plate should be read in the PHERAstar *FS*.

PHERAstar *FS* instrument settings

Measurement type:	Luminescence
Measurement mode:	Endpoint
Optic module:	LUM plus module
Gain:	3600
Measurement time:	1.0 seconds (0.2 seconds for Proof-of-Concept Screen and Counter Screen)

Results & Discussion

An enzyme titration was performed in order to find a useful enzyme concentration for screening. The enzyme titration measurement was started immediately after addition of developer reagent and data points were collected every 2 min for 46 min. After 20 min a plateau was reached (data not shown). Based on these findings all further microplates were prepared using 5 nM of enzyme and incubated for 10 min at room temperature before the measurement started.

Rev. 12/2013
Keywords: **Active compound screening, Dose response, HDAC, Histone, Proof-of-concept screening,**

Dose-response curve of a standard inhibitor

To test the assay for a standard inhibitor, different concentrations of inhibitor were prepared and measured. The results can be seen in Fig. 2.

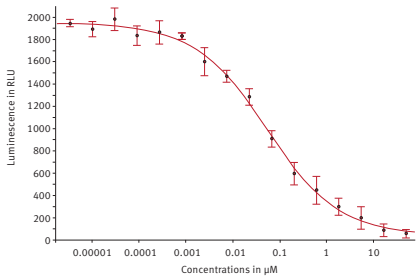

Fig. 2: Dose-response curve for HDAC inhibitor (Trichostatin A).

A 4-parameter fit was applied in the MARS software. The resulting IC_{50} value was 55 nM and agreed with our experience for that standard inhibitor.

Proof-of-Concept Screen and Counter Screen

A subset of the LOPAC library was screened for active compounds against HDAC. The screening results in the plate were visualized by setting limits in the MARS software. The results can be seen in Fig. 3.

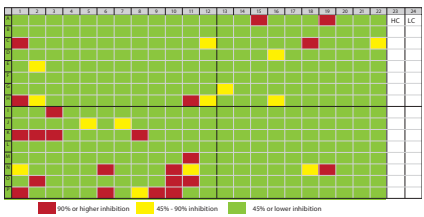

Fig. 3: Results for the HDAC Proof-of-Concept Screen. Wells of column 23 and 24 are control wells (HC = high control, LC = low control).

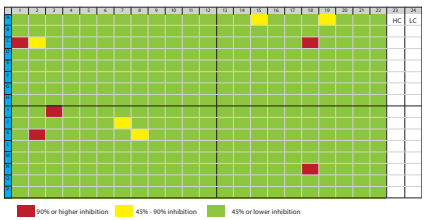

Fig. 4: Results for HDAC Counter Screen. Well of column 23 and 24 are control wells (HC = high control, LC = low control).

Active compounds against HDAC will inhibit HDAC activity. The evaluation of the data is done with the MARS Data Analysis Software and based on the % inhibition calculation using the following formula:

$$\% \text{ inhibition} = 100 - \frac{100}{\textit{average of high control}} * \text{sample}$$

Most promising Hits are defined to show 90 % or higher inhibition (red marked wells in figure 3).

A few Hits were found during the compound screen. Some of them showed also up in the Counter Screen, indicating false negatives that are either active against the protease in the developer solution or against the luciferase. This shows that Counter Screens are very useful especially if the assay includes a coupled enzyme system to detect target activity.

Conclusion

This application note shows that the PHERAstar *FS* can be used for screening assays as well as for assay development. The data is obtained quickly and reliably. Hits can be identified even during the measurement using the current state option. Data processing and color gradient microplate views can be easily adapted using the MARS Data Analysis software.

Detecting Sclerostin-LRP5/6 interaction using the CLARIOstar® and LVF Monochromator™

Carl Peters[1] and Courtney Noah[2]
[1] BMG LABTECH [2] Enzo Life Sciences

- **Enzo's Leading Light® Sclerostin-LRP Screening System is a novel tool to identify Wnt-pathway modulators**
- **The CLARIOstar® can detect this assay using the LVF monochromator™**
- **Excellent Z' values are indicative of the robust assay and sensitive detection**

Introduction

Bone remodeling is the process where bone is continually removed and replaced. Disruption of this process can lead to osteoporosis, characterized by low bone mineral density or bone thickening characteristic of sclerosterosis. Bone remodeling involves the actions of two cell types found in bone, osteoblasts (bone resorption) and osteoclasts (bone deposition). The actions of these two cell types is coordinated by a third type of bone cell called an osteocyte. Sclerostin has now been identified as a molecule expressed by osteocytes that is able to modulate the Wnt-signaling pathway which is important in regulation of bone formation.
Wnt exerts its effect on bone formation by binding to the LRP 5/6 – Frizzled receptor on osteoblasts. This leads to stabilization of intracellular β-catenin and regulation of transcription that promotes bone formation. By binding to the LRP 5/6 receptor sclerostin antagonizes Wnt-signaling and inhibits bone formation. Therefore treatments which block the sclerostin LRP 5/6 interaction could serve as treatments for osteoporosis. In this application note we show that the CLARIOstar microplate reader from BMG LABTECH can be used to screen for Sclerostin-LRP5/6 binding inhibitors.

Assay Principle

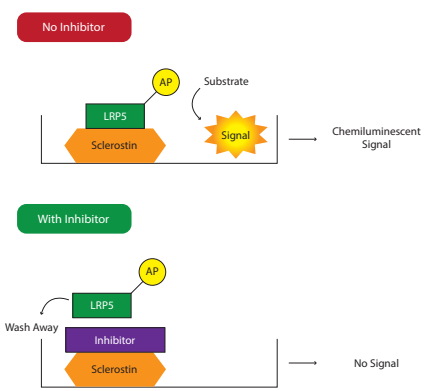

Fig. 1: Sclerostin-LRP System Assay Principle In the absence of binding inhibitors LRP5-AP binds to the Sclerostin with which the 96-well plate is coated. Therefore a chemiluminescent signal is produced upon activation of the enzyme. In contrast, the presence of inhibitors leads to decreased LRP5-AP binding which is washed away. As a result no enzyme activity is detected.

The Leading Light™ Sclerostin-LRP Interaction Screening System uses LRP5 engineered such that it is linked to the enzyme alkaline phosphatase (AP). Sclerostin is coated on 96-well plates and bound LRP5-AP is detected by the activity remaining after a washing step (Figure 1). In this way a variety of different compounds can be screened for their ability to disrupt the sclerostin/LRP5 interaction. Since this is a biochemical assay there is no requirement for a cell line or the stable or transient transfection of a reporter gene.

Materials & Methods

- CLARIOstar microplate reader from BMG LABTECH
- Leading Light™ Sclerostin-LRP Interaction Screening System kit (ENZ-61003) from Enzo® Life Sciences
- white 96-well microplate, Costar

Reagents and microplate supplied in the Sclerostin-LRP Screening kit were prepared according to the procedures described in the product manual. The kit contains a positive control (LRP5-AP fusion protein), a negative control (AP concentrate only) and an inhibition control (Acid Green 25). All plate shaking steps employed the CLARIOstar orbital shaking feature. Upon finishing assay preparation and washing steps the alkaline phosphatase substrate was added.

After a 25 minute incubation the chemiluminescence signal was measured in the CLARIOstar.

To determine appropriate monochromator settings an emission scan was performed using the following instrument settings:

Measurement Method:	Luminescence
Reading Mode:	Spectral scan
Emission wavelength:	500 – 600 nm
Emission bandwidth:	20 nm
Gain:	3600
Focal height:	11.0

The plate was then read in the CLARIOstar with the following adjustment.

Measurement Method:	Luminescence
Reading Mode:	Endpoint
Measurement interval time:	1 second
Emission wavelength:	550.0 nm
Emission bandwidth:	various
Gain:	3600
Focal height:	11.0

Rev. 07/2014
Keywords: **Alkaline phosphatase, Bone, Chemiluminescence, Sclerostin, Wnt pathway**

Results & Discussion

The CLARIOstar spectral scan feature allows you to obtain an image of the luminescence emission of your lumiphore at resolution of 1 nm (Figure 2).

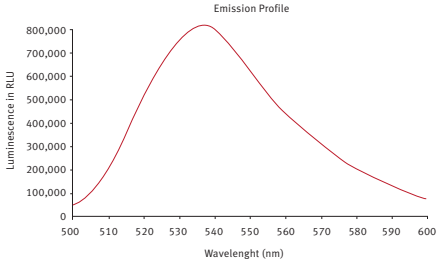

Fig. 2: Emission spectrum for the reaction of alkaline phosphatase with its substrate in the Leading Light® Sclerostin-LRP Screening Assay.

Based on the broad emission spectrum in this experiment we believed that capturing the entire emission spectrum from 500 nm to 600 nm will give best results. The LVF Monochromator in the CLARIOstar makes detecting bandwidths up to 100 nm possible. Using the monochromator setting of 550-100 we were able to collect light from this entire spectral range. Excellent results in the assessment of the inhibitory capacity of Acid Green 25 on the interaction between sclerostin and LRP5 were obtained (Figure 3).

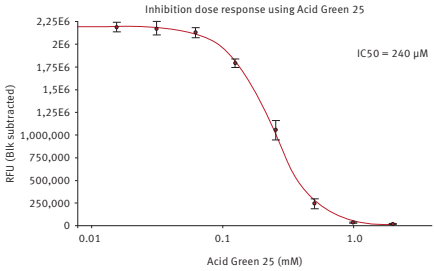

Fig. 3: Acid Green 25 inhibition data, plotted in a 4-parameter fit using the MARS Data Analysis software (R2 = 0.9998). From the inhibition response curve an IC50 value of 240 µM was calculated.

Table 1 provides a comparison of various mono-chromator settings to assess the effect of decreasing bandwidth on assay performance parameters.

Bandwidth (550-)	100 nm	90 nm	80 nm
Avg. PC*	2,216,000	2,022,000	2,004,000
Assay window	275	254	248
Z'	0.972	0.968	0.968

Avg. PC* = average luminescence value of positive control

As we would expect decreasing the bandwidth leads to a decrease in RLU which correlates with a slight decrease in assay window. The assay window is the ratio of positive control and negative control. The effect on Z', however, is negligible.

Conclusion

The CLARIOstar LVF Monochromator exhibits robust luminescent detection sensitivity for Enzo's Leading Light® Sclerostin-LRP Interaction Screening System. This assay system and instrumentation proves to be an excellent platform to assess modulators of sclerostin-LRP interactions.

CLARIOstar®

Detection of PARP-induced ADP-ribosylation using a BMG LABTECH microplate reader

A. Thorsell, A. Pinto, T. Ekblad and H. Schüler
Karolinska Institute, Department of Medical Biochemistry and Biophysics, Stockholm, Sweden

- **PARP enzyme activity determined using a chemiluminescence assay**
- **The luminescence readout is reproducible and linear over a wide enzyme concentration range**
- **The kinetic parameter K_M and the IC_{50} value for an enzyme inhibitor were calculated**

Introduction

PARP (Poly(ADP-ribose) polymerase) family enzymes are involved in the regulation of transcription, DNA repair, and chromatin remodeling. These enzymes use nicotinamide adenine dinucleotide (NAD) as a substrate to build poly(ADP-ribose). Due to various links to diseases, PARP enzymes are targets for pharmaceutical drug development.
In this application note we describe the use of a chemiluminescent assay to determine PARP activity on the CLARIOstar multimode microplate reader. The assay allows kinetic analysis of PARP enzymes and evaluation of inhibitor potency.

Assay Principle

PARP activity is followed *in vitro* by detecting the incorporation of biotinylated ADP-ribose as a consequence of either enzyme target protein modification or auto-modification. The reaction principle is shown in Fig. 1

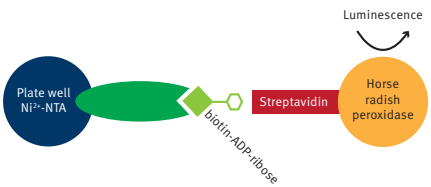

Fig. 1: PARP chemiluminescent assay principle.

Hexahistidine-tagged PARP enzyme or protein substrate is immobilized on Ni^{2+}-chelating microplates. The reaction is started by adding biotinylated NAD^+. The PARP enzyme uses the NAD^+ to synthesize biotinylated poly(ADP-ribose). This polymer is either added to the PARP enzyme itself or transferred to a protein substrate on the microplate (histone). After a washing step streptavidin-conjugated horseradish peroxidase is added and will bind to the biotinylated poly(ADP-ribose). After adding a substrate to the horseradish peroxidase, chemiluminescence is released and can be measured.

Materials & Methods

- Ni-NTA-coated, opaque, white 96-well microplates (5-PRIME)
- Streptavidin-conjugated horseradish peroxidase (Jackson Immunoresearch)
- Biotinylated NAD^+ (Trevigen)
- SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific)
- Microplate reader from BMG LABTECH

All standard chemicals and disposables were obtained through normal distribution channels.

Enzymatic reactions

Hexahistidine-tagged PARP enzyme or protein substrate was immobilized on Ni^{2+}-chelating plates. ADP-ribosylation reactions were started by addition of NAD^+ (2 % biotinylated) at 20°C. Reactions were stopped by addition of 7 M guanidine hydrochloride. Plate wells were washed with reaction buffer, incubated for 30 minutes with TRIS-buffered saline containing 0.02 % Tween-20 (TBST) and 1 % (w/v) BSA, and washed with TBST. After incubation with streptavidin-conjugated horseradish peroxidase (0.5 µg/ml) another washing step was done. After adding SuperSignal West (50 + 50 µl, undiluted) as substrate for the peroxidase chemiluminescence was detected in the CLARIOstar microplate reader using the following instrument settings.

Instrument settings

All measurements (linear range check, K_M determination and inhibitor dose-response) were done in endpoint mode.

Optic:	top optic used
Measurement interval time [s]:	1.00
Presetname:	Enliten ATP
Emission:	full range (no filter)
Gain:	needs to be adjusted prior the measurement
Focal height:	needs to be adjusted prior the measurement

Results & Discussion

For validation of the ADP-ribosyltransferase assay the linear range of signals obtained by a dilution series of biotin-ADP-ribosylated enzyme was determined (Fig. 2). The results show that the signal is linear over a wide range of ADP-ribosyl concentrations.

Rev. 10/2014
Keywords: **HRP, Inhibition dose response, K_m, NAD^+, PARP**

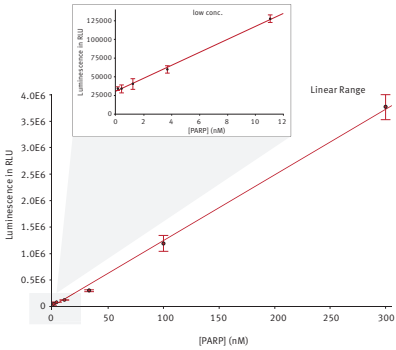

Fig. 2: A dilution series of a PARP-family enzyme under assay conditions, illustrating the linear range of the signal. The insert zooms into the low nM concentration range.

The kinetic parameters of a PARP enzyme family member were determined using initial reaction rates. Independent experiments showed that the biotin moiety linked to the co-substrate had no influence on the reaction kinetics (results not shown).

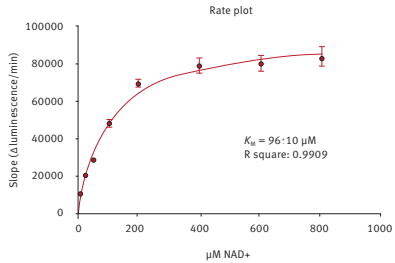

Fig. 3: Rates plot of the NAD⁺-dependent ADP-ribosylation catalysed by a PARP-family enzyme.

Knowledge of K_M allowed the determination of inhibitor dose-response curves and experimental parameters (IC_{50}).

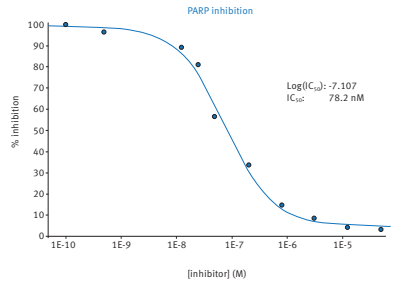

Fig. 4: Dose-response curve for inhibition of a PARP-family enzyme with a clinical PARP inhibitor (Olaparib).

Conclusion

An ADP-ribosylation assay of PARP enzymes carried out in a BMG LABTECH microplate reader shows signal linearity over a wide range of enzyme concentrations (0.015 to 300 nM). The assay allows enzyme characterization and calculation of different parameters that are important for the development of drug like enzyme inhibitors.

PHERAstar® *FSX*
PHERAstar® *FS*

CLARIOstar®

Omega Series

Chemiluminescence measurement of the generation of reactive oxygen species

Bernd Hipler[1] and Uta-Christina Hipler[2]
[1]BMG LABTECH, Jena, Germany [2]Department of Dermatology, FSU Jena, Germany

- **Very low ROS concentrations detectable**
- **Linear correlation between ROS and blastospores concentration**
- **Time- and dose-dependent inhibition of ROS generation by terbinafine confirmed**

Introduction

Phagocytosis is one of the oxygen depending processes in organisms. During the unspecific immunological defense, the activity of pentose phosphate pathway is dramatically increased forming NADPH. NADPH is needed to reduce the oxygen which is bonded to membrane-based cytochromes.
Therefore, the oxygen demand is strongly increased (respiratory burst). During this process, oxygen is converted into superoxide anions, hydrogen peroxide, monomolecular oxygen and hydroxyl c radicals by means of several kinds of phagocytic cells (e.g. neutrophil, eosinophil and basophil leucocytes, macrophages). These extracellular highly reactive oxygen species (ROS) cause many biological effects such as destruction of bacterial cells, parasites and tumor cells, promoting inflammation and modulating the immune reaction (Fig. 1).

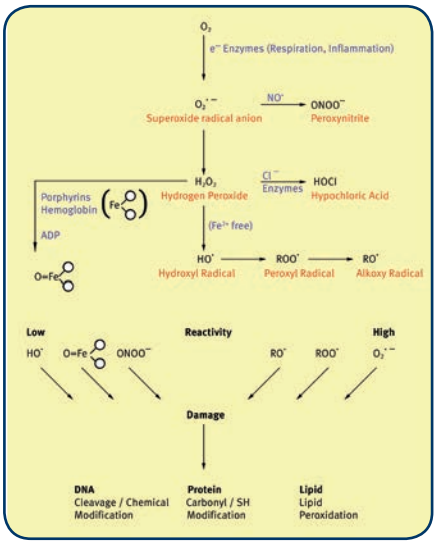

Fig. 1: Reactive oxygen species and oxidative damage.

The generation processes of reactive oxygen species can be monitored using luminescence analysis. Measurements of chemiluminescence (CL) are highly sensitive and specific, owing the possibility to investigate the different kinds of reactive oxygen species simultaneously (HO•, $O_2^{\bullet -}$, H_2O_2, $^1O_2$). Because of the very weak native luminescence phenomena, luminol or lucigenin dependent chemiluminescence has been used frequently for the detection of superoxide radical anions in biological systems. Luminol reacts in its univalently oxidized form and lucigenin reacts in its univalently reduced form with $O_2^{\bullet -}$. In both cases, light production depends on the formation of an unstable endoperoxide or dioxetane, which decomposes to an electronically excited product. This product releases a photon as it falls to the ground state.

In this application note we show the measurement of ROS generation in the yeast *Candida albicans* (*C. albicans*) using lucigenin. In *C. albicans*, mitochondria are capable of generating extracellular released ROS. The effect of terbinafine, a considered free radical interceptor, on ROS formation is investigated.

Materials & Methods

The following strains of Candida were used for the measurement of cellular luminescence: *C.albicans, C.tropicalis, C.guilliermondii, C.glabrata, C.parapsilosis.*

The strains of fungi were cultured 24 h on Sabouraud-Glucose- Agar in the presence of penicillin and gentamicin. Yeast suspensions were prepared in RPMI-1640 medium with concentrations of 10^7 to 10^{11} blastospores/mL or in isotonic NaCl solution with the same concentration. 200 µL of several yeast suspensions were used. Candida cells at a concentration of $1x10^8$ cells/mL were incubated in saline with terbinafine at concentrations of 1 µg/mL, 10 µg/mL and 100 µg/mL dissolved in DMSO. Incubation was performed for 10 min and 60 min at 25°C in a shaking incubator. The duration was restricted to exclude effects of terbinafine on cell number and growth. Lucigenin solution was prepared in PBS at a concentration of 10^{-4} mol/L and added into each well at 30°C or 37°C by an injection pump.

Instrument settings

	FLUOstar®/ POLARstar® Omega	CLARIOstar®	PHERAstar® *FS*
Detection mode	Luminescence		
Method	Plate mode kinetic		
Optic settings	lens	lens	Luminescence Optic Module
Measurement time per data point	1 second		
Measurement time per cycle	60 seconds		
Number of cycles	20		

Rev. 12/2014
Keywords: ***Candida albicans***, **Microbial growth, ROS, Terbinafine, Yeast**

Samples were taken from yeast strains at different cell numbers and for a fixed cell number with varying terbinafine concentration. The calculation was performed using the average counts per minute (cpm) over measurement periods of 20 min.

Results & Discussion

The results of lucigenin dependent chemiluminescence measured in suspensions of *Candida albicans*, *Candida glabrata*, *Candida guilliermondii*, *Candida parapsilosis*, *Candida tropicalis* are shown in Table 1.

Table 1: Comparison of different yeast species.

	Candida albicans	*Candida guillier-mondi*	*Candida parapsi-losis*	*Candida tropicalis*	*Candida glabrata*	*Tricho-sporon capitatum*
cells	RLU	RLU	RLU	RLU	RLU	RLU
E+7/mL	0	0	2	0	0	19
E+8/mL	0	0	4	0	7	94
E+9/mL	2	12	19	25	35	101
E+10/mL	30	48	61	90	126	124
E+11/mL	64	122	156	102	189	324
	r = 0.91 p = 0.02	r = 0.96 p = 0.01	r = 0.960 p = 0.009	r = 0.74 p = 0.10	r = 0.84 p = 0.07	r = 0.970 p = 0.004

(RPMI-medium, T=30°C, Lucigenin-solution 100 µL)

It was notable that the generation of reactive oxygen species in *C. albicans* could not be detected at concentrations of 10^7 to 10^9 blastospores/mL. Detectable CL values were measured at ≥ 10^{10} blastospores/mL. The reproducibility of the results could be demonstrated by inter assay variation as well as linear correlation (r=0.91 resp. 0.98) of the concentration of blastospores and the relative light units (RLU). The linear and direct proportion of the ROS generation and blastospore concentration could be confirmed due to the calculated correlation coefficients. The reproducibility of the results has been demonstrated by inter assay variation on several days (data not shown). After 10 min of incubation with terbinafine, a dose-dependent inhibition of ROS generation was already seen (Fig. 2).

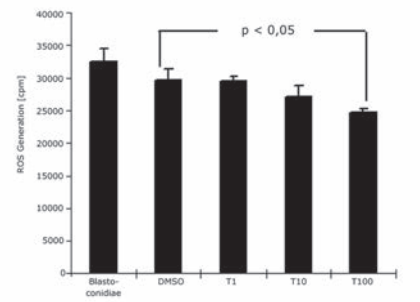

Fig. 2: Dose-dependent inhibition of ROS generation by *Candida albicans* after 10 min incubation with different concentrations of terbinafine. Blastoconidiae cell number: 1 x 10^8 cells/mL in saline. All other samples contain yeast cells and additionally DMSO or terbinafine. DMSO: + 1 % DMSO, T1: + 1 µg/mL terbinafine, T10: + 10 µg/mL terbinafine, T100: + 100 µg/mL terbinafine.

DMSO itself, as the soluble reagent, revealed anti-oxidant properties. However, the inhibition of ROS generation was considerably dependent on the used terbinafine concentration. At a terbinafine concentration of 1 µg/mL (T1) the inhibition corresponded to 9.4 % and at 100 µg/mL (T100) to 24 %.

The inhibition was significant for the comparison of the control incubated with DMSO and the highest chosen terbinafine concentration of 100 µg/ml (T100) ($p<0.05$). After 60 min of incubation with terbinafine the inhibition was amplified (data not shown). The final inhibitory effect was 18 % for 1 µg/mL terbinafine (T1) and 41.1 % for 100 µg/mL terbinafine (T100). The effect was significant for terbinafine concentrations of 10 µg/mL (T10) and 100 µg/mL (T100) ($p < 0.05$). The dose of 10 µg/mL (1 %) corresponds to the dose applied for topical preparations.

Conclusion

The ability of various candida yeasts and blastomyces to generate ROS can be monitored by means of lucigenin-dependent chemiluminescence. The CL method is sensitive enough to detect very low ROS concentrations produced by several fungi without any stimulation.

This was used to look for terbinafine effects on the ROS formation in *C.albicans*. The results confirm a terbinafine time- and dose-dependent inhibition of ROS generation by *C. albicans*. Due to the time-dependency, it is likely that terbinafine not only has radical scavenging properties, but also interacts with the pathway that generates reactive oxygen species. In summary, terbinafine reduces the ability of *C. albicans* to generate reactive oxygen species.

Sequential or simultaneous emission detection for BRET assays

Marjan Orban
BMG LABTECH, Ortenberg, Germany

- **On-board (internal) reagent injectors for high throughput and fast kinetics**
- **A factor of 50 between positive and negative controls**

Introduction

Bioluminescence Resonance Energy Transfer (BRET) is a system of choice for monitoring intermolecular interactions in vivo. BRET is an advanced, non-destructive, cell-based assay technology that is perfectly suited for proteomics applications, including receptor research and the mapping of signal transduction pathways. The assay is based on non-radiative energy transfer between fusion proteins containing Renilla luciferase (Rluc) and e.g. Yellow Fluorescent Protein (YFP). The BRET signal is generated by the oxidation of a coelenterazine derivative substrate.

For this application note the BRET2™ demo kit has been used to prove the feasibility of performing a BRET assay on a BMG LABTECH microplate reader. The BRET demo kit applies the cell-permeable and non-toxic coelenterazine derivative substrate DeepBlueC™ (DBC) and a mutant of the Green Fluorescent Protein (GFP2) as acceptor. These compounds show improved spectral resolution and sensitivity over earlier variants.

Materials & Methods

All materials were obtained through normal distribution channels from the manufacturers stated.

- BRET2™ demo kit, PE Life Sciences
- White 384-well OptiPlate™, PE Life Sciences

Experimentals
A description for the development of BRET2™ protein-protein interaction assays is included with the demo kit. The following section focuses on the microplate reader settings recommended in the assay protocol.

BRET2™ demo kit reagents:
- Non-transfected CHO cell extracts
- Negative control (Rluc + GFP2 not fused together)
- Positive control (Rluc-GFP2 fused together)
- DeepBlueC™
- BRET^{2TM} assay buffer

Assay Protocol (for a white 384-well plate)
1. Addition of BRET2™ assay buffer:
   A10-D12: 15 µL of BRET2™ buffer
2. Addition of 10 µL of each cell extracts (Fig.1):
   A10-A12: Non-transfected cells (blank)
   B10-B12: Neg. BRET2™ control (Rluc + GFP2)
   C10-C12: Pos. BRET2™ control (Rluc-GFP2)
   D10-D12: BRET2™ assay buffer

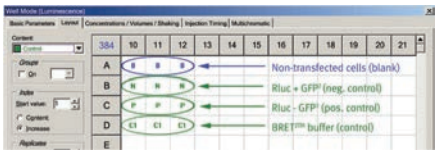

Fig. 1: Layout for the BRET^{2TM} demo kit assay.

3. Automated injection of DBC and measurement:
   Insert the prepared plate in the instrument and fill the injector with DBC solution.
   A10-D12: Injection of 25 µL of DBC at 10 µM
   On-board reagent injectors allow the measurement of high throughput assays and fast kinetic signals. The data from the measurement was evaluated using the MARS Data analysis software.

Instrument settings

	FLUOstar® Omega	POLARstar® Omega	CLARIOstar®	PHERAstar® *FS*
Detection Mode	Luminescence, Well Mode			
Simultaneous dual emission		√		√
Sequential dual emission	√	√	√	√
Filter settings	410-80 515-30	410-80 515-30	410-80 515-30	BRET2 optic module
Gain	3800	3800	3800	3800
Measurement Interval time (seconds)	1.0	0.02	1.0	0.02
No. of. Intervals	50	50	50	50
Injection start time (seconds)	0	0	0	0
Pump speed (µl/s)	260	260	260	260

Results & Discussion

When the donor and acceptor are in close proximity, the energy resulting from the catalytic degradation of the DBC is transferred from Rluc to GFP^2 which will then emit fluorescence at its characteristic wavelength.

The kinetic curves (raw data - blank) of the negative control are shown in Fig.2 for both channels. The low values of the 515 nm channel indicate that no resonance energy transfer occurred. Whereas the positive control shows reduced values at the 410 nm and elevated values at the 515 nm channel due to the BRET effect.

Rev. 12/2014
Keywords: **DeepBlueC, Injectors, GFP2, Protein-protein interaction, Receptor, Signal transduction, Simultaneous Dual Emission SDE**

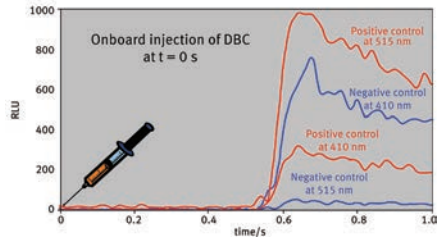

Fig. 2: Resonance energy transfer is obvious for the positive control. No BRET occurs for the negative control. Measurements were performed on a BMG LABTECH instrument using Simultaneous Dual Emission.

The calculated BRET ratio indicates the occurrence of protein-protein interaction *in vivo*. This type of detection eliminates data variability caused by fluctuations in light output which can be found with variations e.g. in assay volume, cell types, number of cells per well and/or signal decay across the plate.

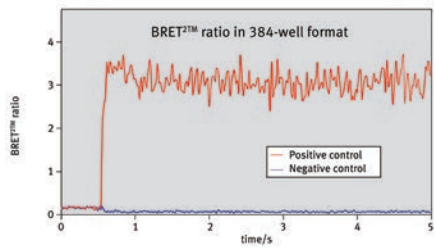

Fig. 3: Ratio of negative and positive control over time. Measurements were performed on a BMG LABTECH instrument using Simultaneous Dual Emission.

In Fig. 3 the blank corrected BRET2™ ratios for both, negative and positive control, are shown and were determined as:

$$\text{BRET}^{2\text{TM}}\ \text{ratio} = \frac{(\text{Em at 515 nm - em at 515 nm of non-transfected cells})}{(\text{Em at 410 nm - em at 410 nm of non-transfected cells})}$$

The MARS Data analysis software offers easy to use tools to do the ratio calculation in just 1 mouse click. The signal for negative and positive control for measurements using simultaneous dual emission reveals a value of around 0.06 and 3.3 respectively, which leads to a factor of around 50 and a clear discrimination between these controls. The signal for negative and positive control for measurements using sequential dual emission lead to values of around 0.05 and 1.9 respectively, which leads to a factor of around 40 (Fig. 4).

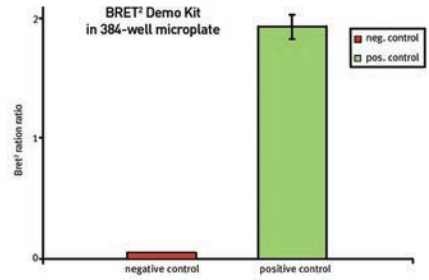

Fig. 4: Ratio of negative and positive control- Measurements were performed on a BMG LABTECH instrument using sequential emission.

The high factor between these controls is caused by the artificial fusion construct of the positive control (Rluc-GFP2) resulting in an extremely high BRET. Real assay samples will presumably result in lower ratios. Nevertheless BRET assays show no photo-bleaching or photo-isomerization of the donor protein, or auto-fluorescence from cells or microplates. Furthermore the large spectral resolution between donor and emission peaks in BRET$^{2\text{TM}}$ (115 nm) greatly improves the signal to background ratio over traditionally used BRET and FRET technologies that typically have only a 50 nm spectral resolution.

Conclusion

Ratiometrically quantifiable BRET$^{2\text{TM}}$ assays have been successfully measured using either sequential or simultaneous dual emission. However, the simultaneous dual emission option will lead to a higher assay window. Next to that, more data points per time can be monitored (50 measurements per second) and the measurement itself is a lot faster as two wavelengths are measured at the same time. The internal reagent injectors for 384-well plate format offer a great advantage for this kind of assay.

PHERAstar® *FSX*
PHERAstar® *FS*

CLARIOstar®

Omega Series

Promega's multiplexed cell viability and apoptosis assays

Tracy Worzella and Brad Larson
Promega Corporation, Madison, WI, USA

- **Multiplexing homogeneous cell-based assays for analysis of different parameters from a single sample well**
- **Monitoring of both luminescence and fluorescence output signals from assay well**
- **Assay miniaturization up to 1536-well format**

Introduction

Today's high-throughput screening facilities face increasing demands to generate more information from their existing compound libraries. One method of obtaining this information is to run assays sequentially, looking at one parameter followed by another in different plates. While this option may produce the desired data, the increased time and consumable costs are drawbacks. A more appealing method for data generation is to perform assays in a multiplexed format in which several parameters can be measured within the same well. This multiplexed format not only saves time and consumable cost, but also saves on the usage of valuable test compounds.

This concept of assay multiplexing is demonstrated here using several cell-based assays multiplexed together. There are inherent properties to cell assays that make them attractive for multiplexed cell-based applications. Cell-based assays are especially vulnerable to variations due to differences in cell growth and metabolism that can arise from plate-to-plate. Cell culture itself is also expensive. By multiplexing assays, fewer cells are needed to acquire the same amount of data. Using the same cells for subsequent assays can also ensure more precise data. In this application note, we demonstrate the combination of several Promega cell-based assays multiplexed in both low-volume 384- and 1536-well plate formats. Table 1 + 2 highlight the assays used in this application note.

Table 1: Cell-based assays for multiplexing applications, set 1.

Assay/ Detection mode	Assay Description
CellTiter-Glo® Luminescence	Cell viability based on the quantification of ATP contained in viable cells in culture. Luminescence is directly proportional to the number of viable cells.
EnduRen™ Luminescence	*Renilla* luciferase reporter luminescence via a protected coelenterazine substrate designed to generate *Renilla* luminescence from living cells. Once inside the cell, the protective groups of the substrate are cleaved by intracellular esterases, generating coelenterazine which reacts with *Renilla* to produce light. Peak luminescence is achieved after 1.5 hours of substrate addition to cells, and signal is stable for > 24 hours.
ViviRen™ Luminescence	*Renilla* luciferase reporter luminescence via a protected coelenterazine substrate designed to generate *Renilla* luminescence from living cells. Once inside the cell, the protective groups of the substrate are cleaved by intracellular esterases, generating coelenterazine which reacts with *Renilla* to produce light. Peak luminescence is achieved after 2 minutes of substrate addition to cells, with signal half-life from 8 – 15 minutes.

Table 1: Cell-based assays for multiplexing applications, set 2.

Assay/ Detection mode	Assay Description
Caspase-Glo® 3/7 Luminescence	Activity of caspase-3 and caspase-7 in cells undergoing apoptosis via cleavage of a Z-DEVD-luciferin derivative. Luciferin reacts with luciferase, ATP and oxygen to produce light. Light output is directly proportional to caspase activity.
Apo-ONE® Fluorescence	Activity of caspase-3 and caspase-7 in cells undergoing apoptosis via cleavage of a Z-DEVD-R110 substrate. The R110 leaving group becomes intensely fluorescent. R110 fluorescence is directly proportional to caspase activity.
CellTiter-Blue® Fluorescence	Cell viability based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Viable cells retain the ability to reduce resazurin into resorufin, whereas non-viable cells lose this ability. Fluorescence is directly proportional to the number of viable cells.

Materials & Methods

- Dedicated Promega assay kits
- Corning low-volume 384-well plates
- Corning 1536-well plates

Multiplexing Cell Viability and Apoptosis Assays

Promega's fluorescent CellTiter-Blue® cell viability assay was multiplexed with either the luminescent Caspase-Glo® 3/7 assay, or the fluorescent Apo-ONE® assay.

Multiplexing Luciferase Reporter and Cell Viability Assays

Promega's luminescent CellTiter-Glo® assay was multiplexed with either the luminescent EnduRen™ Live Cell Substrate, or the luminescent ViviRen™ Live Cell Substrate.

Assay Miniaturization to 1536-well format

For the 1536-well assay format, a density of 4,000 stably transfected cells per well was plated with the Deerac Fluidics Equator. The multiplex protocols were performed identically to the low-volume 384-well (more detailed information on the methods can be found at www.bmglabtech.com).

Results & Discussion

Multiplexing Cell Viability and Apoptosis Assays

Figures 1 and 2 show the results of two experiments to determine the method of cell death caused by different concentrations of anti-FAS antibody in Jurkat cells. The two experiments measured two different endpoints:

Rev. 12/2014
Keywords: **Apoptosis, Caspase-Glo 3/7, CellTiter-Glo 3/7, Cell viability, Multiplexing**

reduction of a resazurin dye as an indicator of viable cells and caspase activity as a marker for apoptotic cells. The data show that with increasing concentration of anti-FAS antibody, an increase in caspase-3/7 activity, with a corresponding decrease in cell viability, is observed.

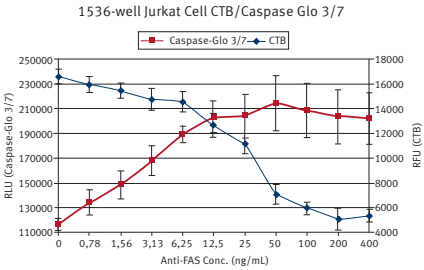

Fig. 1: Sequential multiplexing of a fluorescent cell viability (CellTiter-Blue®) with a luminescent apoptosis assay (Caspase-Glo® 3/7) in 1536-well format.

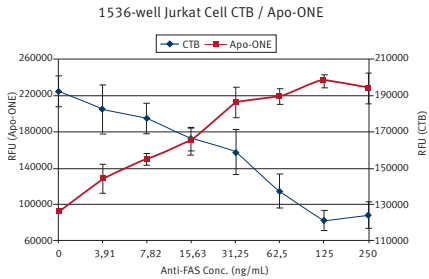

Fig. 2: Sequential multiplexing of a fluorescent cell viability assay (CellTiter-Blue®) with a fluorescent apoptosis assay (Apo-ONE®) in 15356-well format.

The results suggest that the cell population studied is less viable over the range of treatment due to an increase in apoptosis, as opposed to necrosis. For all cell viability and apoptosis multiplexing combinations, results in 1536-well format are comparable to results in 384-well format.

Multiplexing Luciferase Reporter and Cell Viability Assays

To correlate Renilla luciferase reporter gene signal for cell viability, Promega's CellTiter-Glo® assay was multiplexed with either the EnduRen™ Live Cell Substrate or the ViviRen™ Live Cell Substrate. After luminescence reporter signal determination, the CellTiter-Glo® reagent was added at each measurement point to inactivate *Renilla* luminescence and initiate ATP-dependent luminescence, which was recorded to measure the cell viability (Fig. 3 and 4).

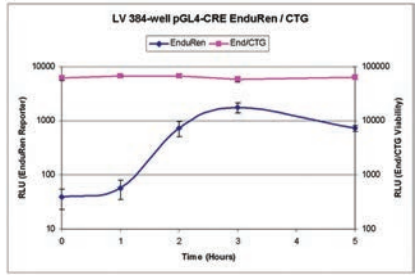

Fig. 3: Coupled *Renilla* reporter activity with cell viability in 384-well format. The kinetic profile of a *Renilla* reporter gene is correlated with the overall cell number.

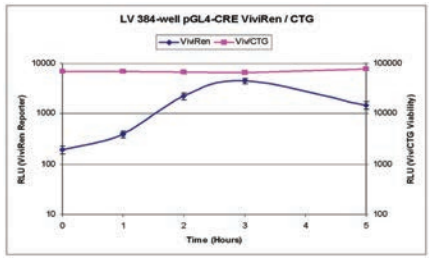

Fig. 4: Coupled *Renilla* reporter activity with cell viability. *Renilla* luminescence is first recorded. CellTiter-Glo® Reagent is added, followed by luminescence reading in 384-well format.

Using live cell reporter substrates, it is possible to track the response of a *Renilla* reporter in real time. Including a cell viability assay allows one to correlate reporter response with overall cell number.

Conclusion

Data from miniaturized assays in 1536-well format are comparable to those run in low volume 384, indicating that smaller assay volume does not compromise the results obtained in higher density formats.

Dual Luciferase Reporter (DLR) assay certification

E.J. Dell and Carl Peters
BMG LABTECH

- **Promega´s DLR assay had been validated on the Omega, CLARIOstar® and PHERAstar® *FS* microplate readers from BMG LABTECH**

Introduction

The Dual-Luciferase® Reporter Assay or DLR is widely used to study gene transcription and regulation. The DLR assay is a two step reaction that uses two luciferase enzymes, Firefly and *Renilla* (Figure 1). The Firefly reaction is initiated, followed by its quenching and the subsequent initiation of the *Renilla* reaction.
The dual measurement of these two enzymes allows for an experimental measurement and a transfection control measurement to be done at the same time. This dual reporting of each sample allows a quantitative result based on the normalization of the *Renilla* luciferase (transfection control). More information on the DLR assay and its certification requirements are available on Promega's website at www.promega.com and in the technical manual.

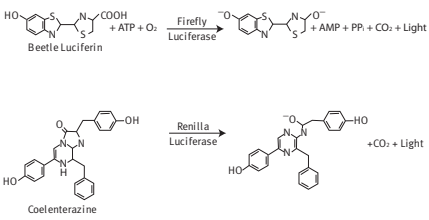

Fig. 1: Bioluminescent reactions of Firefly and *Renilla*.

The certification process consists of 3 parts: Quenching, consistency and tubing adsorption.

The first criterion is the quenching experiment. This will indicate whether the Stop and Glo® reagent, added in injection step 2, has successfully quenched the Firefly luciferase reaction initiated by Luciferase Assay Reagent II which was added in injection step 1.
The second criterion is the consistency experiment. These experiments determine whether a relative standard deviation of less than 5% (%CV) can be maintained by the instrument at two different concentrations of Firefly and *Renilla* luciferase. The tubing adsorption experiment is the third criterion. This experiment shows whether over time the tubing used in the instruments injectors will have an effect on the DLR assay.

These 3 experiments were conducted on the POLARstar®, FLUOstar® and LUMIstar® Omega as well as on the CLARIOstar and PHERAstar *FS* which achieved DLReady™ certification.

Assay Principle

The dual luciferase assay is a fast reaction with 2 injection steps, one for the Firefly substrate (Luciferase Assay Reagent II or LAR II) and one for the Stop and Glo® buffer which contains the Firefly quencher and the *Renilla* substrate (Figure 2).

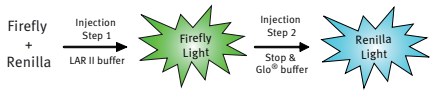

Fig. 2: Dual Luciferase Reaction - Luciferase Assay Reagant II (LAR II) is injected in the first step and the Firefly reaction is started. Stop and Glo® buffer is injected in the second step, which quenches the Firefly reaction and initiates the *Renilla* reaction.

The reaction requires an injection and a measurement for 12 seconds (to quantitate the Firefly luminescence) and then another injection and another 12 second measurement (to quantitate the *Renilla* luciferase).

Materials & Methods

- White, flat-bottom 96-well Costar® plates
- Promega's DLR certification kit
- Recombinant Firefly and *Renilla* luciferase provided by Promega

Instrument settings

	Omega series	CLARIOstar	PHERAstar *FS*
Detection mode	Luminescence		
Method	Well Mode Kinetic, Top optic		
Optic settings	Emission: lens	Emission: full range or Monochromator (520 – 620 nm)	Lumine-scence Optic module
Positioning delay	0.2 seconds		
Number of intervals	48		
Inverval time	0.5 seconds		
Injection start time	0 and 12 seconds		
Injection speed	220 or 230 µl/second		

Rev. 12/2014
Keywords: **DLR, Dual luciferase reporter, Firefly, Injectors, Promega, *Renilla***

These experiments were performed as described in the Promega Instrumentation Certification documentation. Each test varies slightly from running the kit as a whole. Each of the 3 criteria for certification were run according to Promega's guidelines.
For data calculation, the relative luminescence units are summed over two ranges:

Range 1 - Firefly luminescence (3.0-12 seconds).
Range 2 - *Renilla* luminescence (14.5-23.5 seconds).

Results & Discussion

Criterion 1: Quenching of >10,000 Firefly/*Renilla*
Recombinant firefly luciferase exhibited quenching that was >10,000 fold (DLR requirements) (Figure 3). This was calculated by dividing blank corrected Firefly luminescence by blank corrected *Renilla* luminescence (no *Renilla* was used in this experiment).

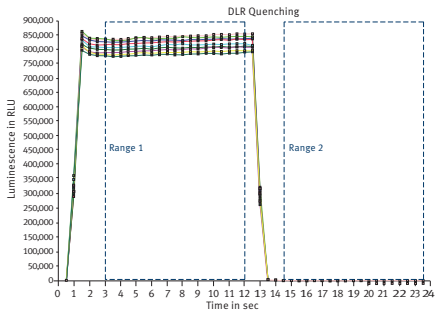

Fig. 3: **Criterion 1**- Graph showing Firefly luciferase quenching taken from MARS evaluation software (>10,000 fold [n=24]. Data measured by the POLARstar Omega using 3.05 ng/ml of recombinant Firefly luciferase.

Criterion 2: Consistency showing < 5% CV
For criterion 2, a 15 X Firefly to *Renilla* luciferase concentration was used for part 1, while a concentration of 30 X *Renilla* to Firefly was used in part 2. The %CV values were below 5 % for all tested BMG LABTECH instrumentation.

Table 1: **Criterion 2** – Consistency has to be < 5 %.

%CV, n = 24	Consistency Part1		Consistency Part2	
	Firefly	*Renilla*	Firefly	*Renilla*
Omega series	2.3	2.5	2.2	2.0
CLARIOstar	2.0	1.5	0.5	2.3
PHERAstar *FS*	1.5	1.8	1.6	0.6

Criterion 3: Tubing Adsorption show < 5% CV after 10 minutes
Similar to criterion 2 part one; 15 X Firefly to *Renilla* was used for this test. Twelve replicates were run followed by twelve more replicates with an intervening 10 minute wait to test for possible tubing adsorption. As with the other tests the % CVs are less than 3 and therefore clearly within the criterion (Table 2).

Table 2: **Criterion 3** – Tubing Adsorption shows little change after 10 minutes, for n=12.

Omega	Firefly		*Renilla*	
	Average	%CV	Average	%CV
RLU	1.055E7	2.5	3.711E6	1.8
RLU (after 10 min)	1.030E7	1.9	3.793E6	2.5
CLARIOstar	**Firefly**		***Renilla***	
	Average	%CV	Average	%CV
RLU	7.006E6	0.7	1.768E5	1.4
RLU (after 10 min)	6.91E6	0.5	1.762E5	2.0
PHERAstar *FS*	**Firefly**		***Renilla***	
	Average	%CV	Average	%CV
RLU	1.552E7	1.5	8.925E5	1.7
RLU (after 10 min)	1.536E7	1.1	1.536E7	1.8

Conclusion

The Omega series of microplate readers as well as the CLARIOstar and the PHERAstar *FS* from BMG LABTECH has been granted DLReady™ certification based on the results published in this application note.

Monitoring intracellular Ca^{2+} fluxes and cAMP with primary sensors from Lonza

Bodo Ortmann[1], Leon de Bruin[1] and A. Pitt[2]
[1]Lonza Cologne GmbH [2]Promega Corporation

- **Luminescence based calcium measurements in HUVECs utilizing Clonetics™ primary sensors**
- **cAMP was detected in hMSC with the help of Poietics™ primary sensors**
- **BMG LABTECH luminescence reader used to monitor dose-dependent responses**

Introduction

Primary cells allow for a higher predictability of drug reactions in humans. These cells endogenously express relevant drug targets at physiological level and genuinely carry the components required for specific signal transduction. They can be derived from the actual tissue of interest. These are significant advantages over immortalized cell lines, which may be derived from irrelevant tissue, be of nonhuman origin, and often express transfected drug targets at non physiological levels. For these reasons, there is a growing demand for primary cells in drug screening and hit validation.

In this application note we show that primary human mesenchymal stem cells can be used in high-throughput formats (i.e. 96-well and 384-well plate) to monitor changes of intracellular cAMP concentration. For the monitoring of Ca^{2+} fluxes human umbilical vein endothelial cells (HUVEC) proved to be useful.

Assay Principle

cAMP Biosensor assay

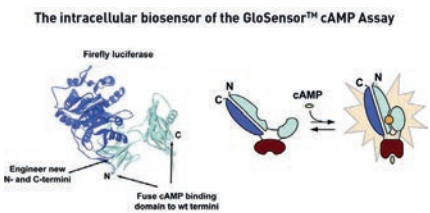

Fig. 1: Mechanism of the GloSensor™ reaction.

Firefly luciferase has been fused genetically to the cAMP-binding domain of human Protein kinase A (red). Upon binding of cAMP the whole protein molecule undergoes a conformational change. This activates the luciferase domain which converts luciferin to oxyluciferin and emits luminescence. For more information see www.promega.com/glosensor.

Calcium Biosensor assay

Incubation of cells, expressing the i-PhotinaR apo-photoprotein, with coelenterazine in the presence of oxygen leads to formation of a stable complex, the active photoprotein. Calcium released from intracellular stores upon stimulation of the cells with agonists via G-protein coupled receptors binds to the photoprotein. The excited photoprotein converts coelenterazine to coelenteramide and emits a flash of blue luminescence.

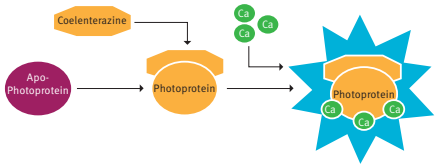

Fig. 2: Mechanism of the i-Photina® reaction.

Materials & Methods

- 96-well and 384-well white microplates from Corning
- pGloSensor™22F cAMP plasmid & GloSensor cAMP reagent from Promega
- Poietics™ human mesenchymal stem cells (hMSC) from Lonza
- Ionomycin, ATP, thrombin, histamine from Sigma
- Neurotensin was from Bachem
- Clonetics™ primary sensors - HUVEC calcium bio-sensor from Lonza

Production of hMSC cAMP biosensor

Poietics™ human mesenchymal stem cells (hMSC) were transiently transfected with an expression plasmid encoding the GloSensor™- 22F using the Amaxa™ 96-well Shuttle™ Nucleofector™. The transfected hMSCs were incubated after Nucleofection™ in a humidified tissue culture incubator (37°C, 5 % CO_2) for 6 hours. Subsequently the cells were frozen in vials in cryoprotective agent.

Production of HUVEC calcium biosensor

Clonetics™ HUVEC were transiently transfected with an expression plasmid encoding i-Photina® using the appropriate Amaxa™ 96-well Nucleofector™ kit and the Amaxa™ 96-well Shuttle™ Nucleofector. The transfected HUVECs were incubated after Nucleofection™ in a humidified tissue culture incubator (37°C, 5 % CO_2) for 6 hours. Right before freezing the cells were loaded with 10 µM native coelenterazine for 2 hours. Subsequently the cells were frozen in vials in cryoprotective agent.

Detection of intracellular calcium release or intracellular cAMP

In order to perform the Ca^{2+}-assay or the cAMP assay cryopreserved cells were thawed, seeded on a 96-well or 384-well plate, and were allowed to recover overnight. 4 hours after thawing medium was exchanged for HEPES buffered medium to remove the cryoprotective agent. The plate was then ready to be measured.

Rev. 12/2014
Keywords: **Biosensor, cAMP, Calcium, Calcium flux, Injectors, Primary cells**

Instrument settings

Calcium assay			
	POLARstar®/ FLUOstar®/ LUMIstar® Omega	CLARIOstar®	PHERAstar® *FS*
Detection mode	Luminescence, plate mode		
Method	Endpoint, Top optic		
Optic settings	Lens	monochro-mator full range	luminescence specific Optic module
Cycle time	5 min		
No of cycles	5-6		
Integration time	5 sec		
Temperature	25°C		

cAMP assay			
	POLARstar/ FLUOstar/ LUMIstar Omega	CLARIOstar	PHERAstar *FS*
Detection mode	Luminescence, well mode		
Method	Endpoint, Top optic		
Optic settings	Lens	monochro-mator full range	luminescence specific Optic module
Temperature	25°C		
Injection	25 µl (384-well), 50 µl (96-well)		
Injection speed	150 µl/sec		
Meas. time/well	35 sec		
Interval time	1 sec		
Integration time	0.7 sec		

Results & Discussion

Ca^{2+} Assay

The reaction of the flash luminescence was followed over time (Fig. 3). Injecting the stimulating agonist while recording of luminescence intensity is on-going. Calcium release was triggered by injection of histamine (50 µM final concentration).

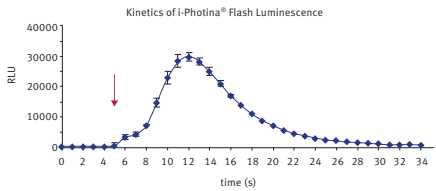

Fig. 3: Kinetics of HUVEC calcium biosensor flash luminescence upon stimulation with 50 µM histamine. The arrow indicates the time point of injection. The assay was performed in 96-well format.

The assay system is well suited to generate pharmacologically relevant data for compounds that trigger calcium dependent signalling. Agonists for different classes of GPCRs (histamine receptors, purinergic receptors, neurotensin receptors) clearly show dose-dependent responses with the HUVEC calcium biosensor yielding EC50 values consistent with published data (data not shown).

cAMP Assay

The functional expression of the GloSensor™ protein in hMSC reactivated from frozen state was demonstrated by treating the cells with forskolin. Result is a clear dose-dependent response with an EC50 consistent with published data (Fig. 4).

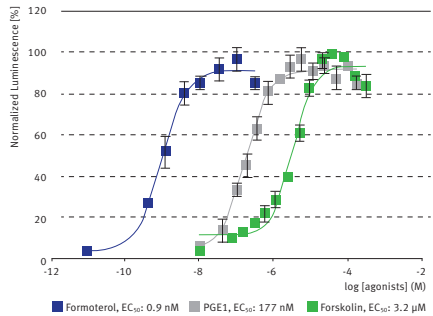

Fig. 4: Dose-dependent responses of hMSC cAMP biosensor to different stimulants.

Conclusion

The ready-to-use cell based assay systems from Lonza in conjunction with a microplate reader from BMG LABTECH are an excellent tool to evaluate drug effects on signaling in primary cells.

Detecting Mycoplasma cell culture contamination using the CLARIOstar® microplate reader

Jacob Serfass[1], Megan Young[1], and Carl Peters[2]
[1]Penn State Hershey College of Medicine [2]BMG LABTECH, Cary, NC

- **MycoAlert™ enables detection of cell culture contamination by Mycoplasma**
- **The CLARIOstar® microplate reader provides sensitive detection of this luminescent assay**
- **Multiple samples can be assessed for contamination in each assay**

Introduction

Mycoplasma is a genus of small bacteria that lack a cell wall around their membrane[1]. They are well characterized as a contaminant of laboratory cell cultures. Their small size makes them difficult to detect and their lack of cell wall makes them resistant to many common antibiotics.
Mycoplasma contamination can cause serious harm to cell cultures. They have been shown to induce a variety of cellular changes and in severe cases can destroy a cell line[2]. The MycoAlert™ Assay is a quick and relatively easy way to detect Mycoplasma contamination. Here we show the utility of the CLARIOstar microplate reader to perform sensitive detection of this assay.

Assay Principle

The MycoAlert™ assay is a biochemical assay that selectively exploits mycoplasmal enzyme activity. Mycoplasma contamination is measured by a bioluminescent reaction that takes place only in viable mycoplasma (Fig. 1).

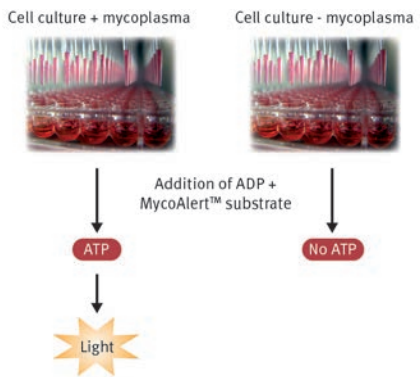

Fig. 1: The MycoAlert Assay employs a selective substrate and detects the ATP produced by mycoplasmal enzymes.

Measurements of luminescence are taken before and after the addition of MycoAlert™ assay substrate. Light production is quantified with a suitable luminometer, such as the CLARIOstar, to observe the presence of light emission a sample may have. Mycoplasma positive samples will produce a high luminescence signal after substrate addition resulting in a ratio of greater than 1 (ratio= reading after substrate addition/ reading before addition).

Materials & Methods

- CLARIOstar from BMG LABTECH
- Lonza MycoAlert™ Mycoplasma Detection Kit (LT07-118)
- Lonza MycoAlert™ Assay Control Set (LT07-518)
- HeLa and MEF cell lines
- Greiner 96 well, F-bottom, white, microplates

MycoAlert™ assay was performed according to kit and assay control set instructions with the exception that 50 µl of samples and controls were employed.

CLARIOstar Instrument settings

Measurement type	Luminescence
Measurement mode	Plate Mode
No. of cycles	15 (pause between 1 & 2)*
Cycle time	60 s
Measurement interval time	1.00 s
Focal height	11.0 mm
Gain	3500

* The assay manufacturer demands that the MycoAlert™ reagent is added manually. This action can be put into the protocol by defining a pause between cycle 1 and cycle 2.

Results & Discussion

As expected, addition of the substrate after the first cycle resulted in a clear increase in luminescence for positive control samples compared to the negative control (Figure 3).

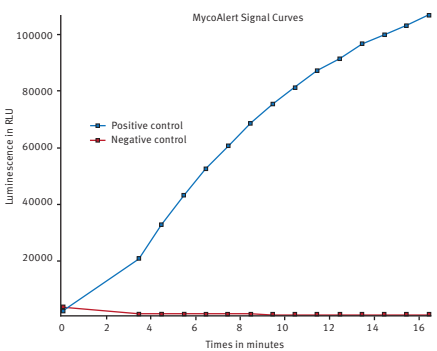

Fig. 3: MycoAlert Signal curve over time. Average (n=3) signal for the MycoAlert reaction in the presence of a MycoAlert positive control sample is shown in blue ( ). Average (n=2) signal for a mycoplasma negative control is shown in red ( ).

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Keywords: **Contamination, Lonza, MycoAlert, Mycoplasma, Validation**

The MycoAlert ratio was calculated in MARS by dividing the luminescence detected at cycle 15 by the luminescence detected at the cycle before addition of the substrate (cycle 1). As you can see in figure 4 decreasing the amount of MycoAlert positive control leads to a decreased ratio as expected.

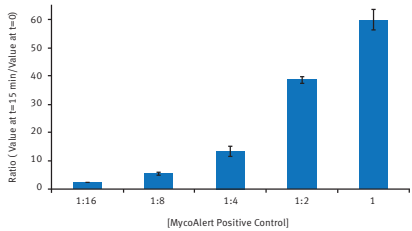

Fig. 4: MycoAlert Ratio vs. positive control amount. The MycoAlert positve control was used to prepare a 1:2 dilution series. The average (n=3) ratio for undiluted or serial diluted samples are shown here.

The MycoAlert protocol states that the negative control should produce a ratio of < 0.9 while positive results should be seen for at least a dilution of 1:8 of the positive control. Using the settings shown here the CLARIOstar readings produced an average ratio of 0.09 for the negative control while the 1:8 dilution of the positive control produced an average ratio of 5.4. In fact even a dilution of 1:16 produced a positive result; 2.5 average ratio.

Finally we used MARS to calculate the MycoAlert Ratio for samples from a variety of cell lines. We used the Validations capability to give a good or bad label depending on whether the ratio was below or above 1. Figure 5 shows how this data can be depicted within the MARS software.

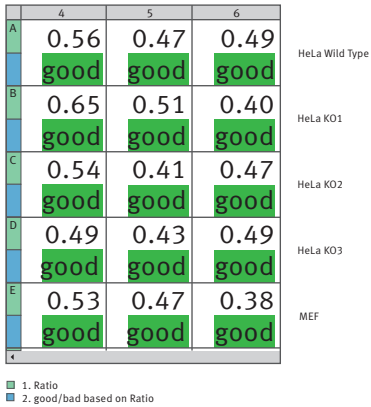

Fig. 5: MycoAlert Ratio calculated in MARS.

Triplicate samples from the indicated cell lines were tested for Mycoplasma contamination. Using MARS data analysis software the MycoAlert Ratio was calculated and could validate if the ratio is less than 1. All tested samples are free from Mycoplasma contamination.

Conclusion

The combination of MycoAlert assay and luminescent detection with the CLARIOstar allows users to quickly and easily check their cell cultures for possible Mycoplasma contamination. MARS calculations and validations allow users to quickly identify samples which may be contaminated.

References

1. S. Razin (1978). *The mycoplasmas*. Microbiol. Rev. **42**: 414-470
2. I.A. Sokolova et al. (1998). *Mycoplasma infection can sensitize host cells to apoptosis through contribution of apoptotic-like endonuclease(s).* Immunol. Cell Biol. **76**: 526-534

Using SPARCL technology to develop immunoassays for biomarker detection and pharmacokinetic studies

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- **The SPARCL assay provides a platform for simplified development of immunoassays**
- **The reading at time of injection capability of the FLUOstar® Omega allows the assessment of the entire SPARCL signal**

Introduction

Biomarkers are used in diverse scientific fields to evaluate normal and pathogenic processes or the response to therapy. Here we look at the biomarker interleukin 8 (IL-8) which is often associated with inflammation and has been shown to be increased by oxidative stress.
Pharmacokinetic (PK) assays are used to measure the concentration of a drug that has been administered to a human or animal. PK assay data are used to evaluate elimination profiles of the drug and are important in drug safety and toxicology studies.
In this application note we will show how the performance of the FLUOstar Omega microplate reader enables the detection of SPARCL (Spatial Proximity Analyte Reagent Capture Luminescence) assays. Examples of biomarker detection and pharmacokinetic assays are presented.

Assay Principle

SPARCL detection technology was used for rapid immunoassay development. This technology is a proximity dependent and homogeneous method where an antibody/antigen interaction leads to proximity of a chemiluminescent substrate (acridan, bound to antibody 1)) and an oxidative enzyme (horseradish peroxidase (HRP, bound to antibody 2)). Introduction of a trigger solution containing H_2O_2 generates a flash of light proportional to the amount of analyte present (Figure 1).

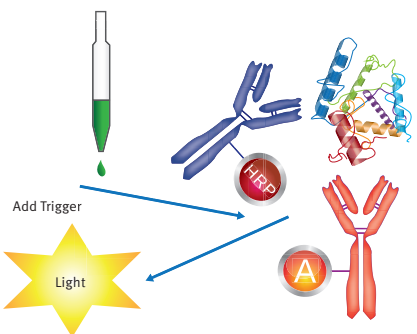


Fig. 1: Representative SPARCL Assay. Specific antibody/antigen interactions bring acridan and HRP into close proximity. The H_2O_2 based trigger solution, which is added subsequently, interacts with acridan (A) and HRP causing a flash of light.

Materials & Methods

- SPARCL kits (Lumigen)
- FLUOstar Omega microplate reader (BMG LABTECH)
- 96-well, white, flat bottom microplates from Greiner
- Other reagents were obtained from commercial sources

A typical Lumigen SPARCL assay workflow is shown below.

Step 1 Label one of the antibodies with the activated SPARCL label.

Step 2 Add antibody solution and sample, incubate for 30 minutes.

Step 3 Add SPARCL background reducing reagent.

Step 4 Read luminescence signals by injecting SPARCL trigger solution on a FLUOstar Omega

For each experiment one antibody was labeled with acridan according to SPARCL kit instructions while a second HRP-labeled antibody was purchased.

Biomarker Assay
A 1:2 dilution series of IL-8 was prepared from a starting concentration of 1000 pg/mL. Dilution of controls and the dilution series were made in cell culture media or PBS containing 0.1% BSA and 10% human plasma.

Pharmacokinetic Assay
A standard curve was generated using a dilution series of human IgG in neat (undiluted) rat serum. The highest point in the standard curve was 1000 ng/mL. Quality Control samples (QC) were made by spiking human IgG drug into neat rat matrix. QC concentration values were interpolated from the standard curve. The interpolated QC sample values were used to determine accuracy, precision and total error.

For both experiments sample plating, antibody addition, incubation and addition of background reducing agent were performed according to instructions.[1] The plates were then read on the FLUOstar Omega plate reader with the following settings which include injection of the trigger solution.

Rev. 06/2015
Keywords: **Biomarker, IgG, IL-8, Interleukin-8, Lumigen, SPARCL**

FLUOstar Omega instrument settings

Measurement type:	Luminescent (well mode)
No. of intervals:	50
Interval time:	0.02 s
Measurement interval time:	0.02 s
Emission filter:	lens
Gain:	3600
Injection volume:	75 µl
Pump speed:	300 µl/s

Results & Discussion

The FLUOstar Omega features reading at time of injection which allows for the assessment of signal produced in the SPARCL reaction upon addition of the trigger solution (Figure 2).

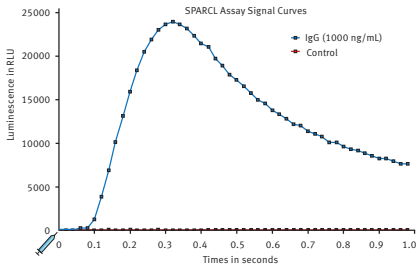


Fig. 2: Typical SPARCL Pattern of Flash Luminescence. Light energy was captured every 0.02 seconds for 1 second. This representative curve contains 1000 ng/mL of human IgG. The control does not contain IgG.

Biomarker Assay

The data produced by the SPARCL IL-8 biomarker experiment was evaluated using results based on calculating the area under the curve. As seen in Figure 3 this method is suitable for assessing the SPARCL reaction providing a reliable fit curve.

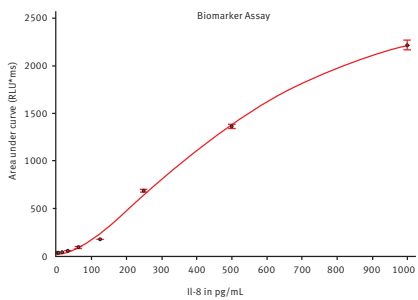


Fig. 3: 4-Parameter Fit Curve of SPARCL IL-8 Data. Data calculated using area under the curve conforms to a 4- parameter fit curve with an R2 value of 0.998.

Pharmacokinetic Assay

Similarly SPARCL PK assays using IgG as a "drug" were performed. Figure 4 shows the representative curve produced from a dilution series of human IgG.

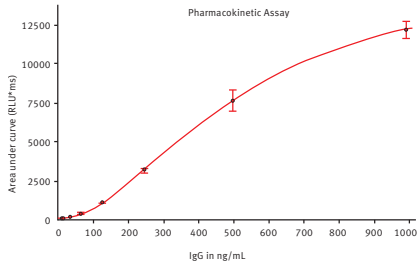


Fig. 4: 4-Parameter Fit Curve of SPARCL PK Data. Data calculated using area under the curve conforms to a 4-parameter fit curve with an R2 value of 0.99996.

Assay precision and reproducibility were assessed for the PK assay using replicates of QC samples tested in 2 different SPARCL assay test runs as shown below (Table 1).

	QC500	QC125	QC31.25
Number of measurements	6	6	6
Accuracy (%RE)*	-0.5	-3.7	-7.6
Precision (%CV)**	8.1	2.9	4.9
Total Error (%RE +%CV)	8.6	6.6	12.5

*%RE = Percent relative error; **CV = coefficient of variation

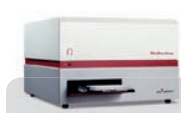
Conclusion

Lumigen's SPARCL assay enables simplified immunoassay development when paired with the reading at time of injection capabilities of BMG LABTECH microplate readers such as the FLUOstar Omega. Both the IL-8 and the PK SPARCL assays show acceptable accuracy, precision and total error. SPARCL may be formatted for many different assay types including high throughput screening applications.

References

1. For more information about SPARCL contact Lumigen at SPARCL@beckman.com or visit www.lumigen.com/detection-technologies/sparcl

PHERAstar® *FSX*
PHERAstar® *FS*



Omega Series

Monitoring receptor ligand binding in living cells

Dominik Schelshorn[1], and Franka Maurer[2]
[1]Geneva Biotech, Switzerland [2]BMG LABTECH, Germany

- **cAMP BRET biosensor system applied to live cells**
- **High sensitive LVF monochromator of the CLARIOstar® microplate reader enables detection of BRET emission signals without using filters**

Introduction

Bioluminescence resonance energy transfer (BRET) is an established method to study protein-protein interactions. Most of the research focuses on GPCRs as these receptors play an important role in finding new drug candidates.[1]
There are several BRET-based approaches that allow the monitoring of protein-protein interaction[2] such as:

- BRET between receptors and ligands[3]
- BRET between receptors and G proteins
- BRET between receptors and downstream effectors, such as adenylyl cyclase or ion channels
- BRET sensors that follow beta-arrestin recrutiment
- cAMP BRET biosensors

In this application we will show how a cAMP BRET biosensor can be used to monitor ligand binding with the help of the CLARIOstar microplate reader.

Assay Principle

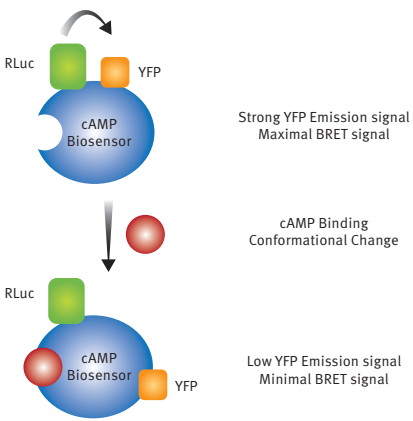


Fig. 1: Assay Principle of detection of ligand binding using BRET1 technology.

The BRET biosensor carries a cAMP binding domain, *Renilla* luciferase and YFP. The latter two are in close proximity. Once the luciferase is activated by substrate addition (Coelenterazine H) the enzyme emission excites YFP. This leads to a strong emission signal at 530 nm and therefore to a strong BRET signal (Fig. 1). Once a ligand is binding to a GPCR, G proteins are activated and cAMP is generated. Free cAMP will find its binding site and the biosensor will undergo a conformational change leading to a greater distance between Rluc and YFP. The result of that process is no or only weak YFP emission leading to a small BRET signal.
Once ligands are found that are able to effectuate the conformational change, a dose response can be run in order to determine EC50 values.

Materials & Methods

- White 96-well microplates from NUNC
- Coelenterazine h from Dalton Pharma Services
- CLARIOstar microplate reader from BMG LABTECH

cAMP biosensors were prepared as described in reference 2. Cells were transiently transfected with the fusion plasmids. One day before BRET experiments the cells were seeded into 96-well microplates.

CLARIOstar settings for ligand binding

Detection Method	Luminescence, plate mode, top reading
Optic setting	Well mode chromatics selected
Monochromator	Just select 475-30 and 535-30 nm, the bandwidth is adjustable up to 100 nm
Filters	Emission filters 475-30 and 535-30 nm
Kinetic settings	No. of cycles: 10 Cycle time: 90 sec Measurement time: 0.50 sec
Gain	3600 for both channels

BRET calculation

- Ratio calculation: 535 nm / 475 nm
- Average of 4 first measurement points (based on ratio) = A0
- Average of 5 last measurement points (based on ratio) = At
- Divide At/A0
- Use color gradient to see positive results immediately (an example is shown in Fig. 3)

Results & Discussion

Initial BRET luminescence scan
Two controls were prepared. One well contained the biosensor without ligand – so a high BRET signal is expected ($BRET_{Max}$ Control). A second well consisted of the cAMP biosensor accompanied by a high ligand concentration. It was a ligand chosen that is known to lead to an increase in cAMP levels and therefore into a conformational change. For the latter a minimal BRET signal is expected ($BRET_{Min}$ Control). Overlayed luminescence spectra are shown in Fig. 2.

Rev. 07/2015
Keywords: **Biosensor, BRET, cAMP, Protein-protein interaction, Receptor ligand binding**

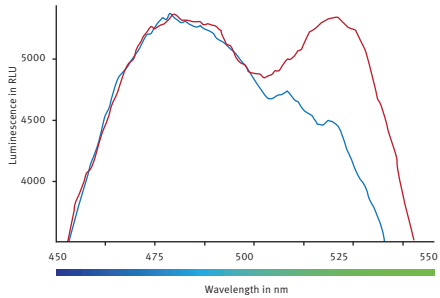


Fig. 2: CLARIOstar LVF monochromator luminescence scan from 320-600 nm with a resolution of 1 nm. The red line corresponds to the $BRET_{Max}$ Control , the blue line is the spectral result of the $BRET_{Min}$ Control. In the MARS Data Analysis software the moving average function was applied (factor of 5) to smooth the curve.

Binding of ligand to receptor

Different ligands can be tested on one microplate. A color gradient was applied in the MARS Data Analysis software to get a fast overview of wells in which the ligand was effectively binding (Fig. 3).

Fig. 3: BRET1 ratio values obtained with the CLARIOstar. A color gradient was applied using green to indicate a successful ligand binding and red to show a negative result.

Only forskolin treated samples showed a decrease in BRET ratio. Forskolin is known to directly activate adenylate cyclase which in turn produces cAMP. Therefore, forskolin can be used as a control substance for this assay. A dose response curve was created by adding varying amounts of forskolin in replicate wells (Fig. 4)

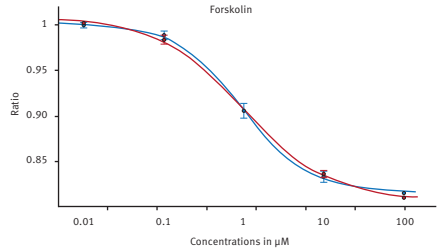


Fig. 4: Forskolin dose response curve obtained with the CLARIOstar. The blue line is based on monochromator measurements while the red line is based on filter measurements.

Although the intrinsic assay window is quite small, the CLARIOstar is sensitive enough to detect small luminescence changes by either using BRET specific emission filters or by using the LVF monochromator. The standard deviation in replicates is small enough to obtain excellent 4-parameter dose response curves with R^2> 0.999.

Conclusion

The CLARIOstar microplate reader is a suitable instrument to detect protein ligand binding with the help of the cAMP BRET biosensor technology. The MARS Data Analysis software can be set up to use a color gradient to fast identify ligands that lead to a receptor conformational change.

References

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2) Salahpour et al. (2012) BRET biosensors to study GPCR biology, pharmacology, and signal transduction. *Front Endocrinol. (Lausanne)* **3**:article105.
3) Stoddart et al. (2015) Application of BRET to monitor ligand binding to GPCRs. *Nat. Methods* **12(7)**: 661-663.

NanoBRET™ assay for monitoring of ligand binding to GPCRs in live cells, using the CLARIOstar® and the PHERAstar® *FS*

Leigh A. Stoddart[1], Elizabeth K. M. Johnstone[2,3], Amanda J. Wheal[1], Joëlle Goulding[1], Matthew B. Robers[4], Thomas Machleidt[4], Giovanni Abbenante[5], Keith V. Wood[4], Stephen J. Hill[1-3], and Kevin D. G. Pfleger[2,3]

- **First use of BRET for monitoring ligand binding to GPCRs in live cells enabled by NanoLuc® luciferase**
- **IC_{50} and K_D values were calculated from saturation and competition binding assays**

Introduction

Due to the crucial role of G protein-coupled receptors (GPCRs) in mediating cellular responses to external stimuli, these receptors have been and will remain a prime focus for medical research and the pharmaceutical industry for many years to come. Their importance is especially highlighted by the statistic that 30-50% of marketed pharmaceuticals target GPCRs.

Current assay techniques for studying ligand binding to GPCRs include use of radioactivity, fluorescent ligands and FRET (fluorescence resonance energy transfer). BRET (bioluminescence resonance energy transfer)[1] requires only the addition of a suitable substrate to generate donor light emission, as well as energy that can excite fluorescently-labelled ligands in a non-radiative manner. The donor luciferase enzyme can be expressed on the N-terminus of the receptor of interest in an appropriate transfected cell line. Theoretically, BRET should be more sensitive than FRET-based assays, however the practical difficulty of finding a suitable luciferase enzyme amenable to such N-terminal fusion has hampered the development of a BRET assay for ligand binding studies.

Recently, researchers at Promega have developed a genetically engineered luciferase called NanoLuc® (Nluc) from a natural luciferase isolated from deep sea shrimp.[2] Nluc is of relatively small molecular weight (19kDa) and is very stable (up to 55°C). Unlike other luciferase-GPCR fusion constructs, it is readily expressed on the N-terminus of GPCRs and efficiently transported to the cell membrane.[3]

Assay Principle

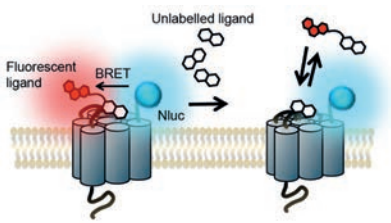

Fig. 1: The NanoBRET™ binding assay principle.

In this particular example, a GPCR is expressed in live cells with Nluc luciferase on its N-terminus.[3] A ligand of this receptor is then labelled with a fluorophore that has an excitation spectrum overlapping the emission spectrum of Nluc. The ligand is introduced to the live cells followed by addition of the Nluc substrate (furimazine). If the ligand does not bind to the receptor, only the blue emission of Nluc is detected. If the ligand binds specifically to the receptor, excitation of its fluorescent label by resonance energy transfer from the Nluc results in red fluorescence also being detected. Competitive binding of unlabelled ligand then causes a concentration-dependent decrease in this red fluorescence (Fig. 1).

Materials & Methods

Detailed materials and methods are provided in the Online Methods of Stoddart et al.[3]

Stable cell line generation: The HEK293 cell line expressing β_2 adrenergic receptor (β_2AR) was from Promega.

Fluorescently labelled ligands: Alprenolol-TAMRA was synthesised by Promega; propranolol-BY630 was obtained from CellAura; alprenolol was obtained from Sigma; isoprenaline, propranolol, ICI 118551 and CGP 12177 were obtained from Tocris.

Nluc and Rluc8 emission spectra: 24 h post-transfection, cells expressing either Nluc-β_2AR or Rluc8-β_2AR were incubated for 180 min at 37 °C in OptiMEM without phenol red (Gibco) in 96 well plates. The luciferase substrates furimazine (Nluc) or coelenterazine h (Rluc8), at a final concentration of 10 µM, were then added to the wells.

BRET β_2AR-ligand binding assays: HEK293 cells transfected with Nluc-β_2AR were assessed in 96 well plates. For saturation binding experiments using alprenolol-TAMRA, serially diluted alprenolol-TAMRA in the absence or presence of 10 µM alprenolol was added to the wells and the plate incubated for 120 min at room temperature. The furimazine substrate was then added to a final concentration of 10 µM. BRET was measured at room temperature. For the competition binding experiments, Nluc-β_2AR stably transfected HEK293 cells were incubated with 10 nM propranolol-BY630 and the required concentration of competing ligand diluted in HEPES-buffered saline solution for 1 h at 37 °C.

Instrument settings

Nluc and Rluc8 emission spectra: The emission spectra were determined with a CLARIOstar® plate reader using the luminescence scanning option of the monochromator (20-nm bandwidth; 1-nm resolution; integration time: 500 ms; gain: 3000).

BRET β_2AR ligand binding assays:

For the saturation binding experiments using alprenolol-TAMRA, the CLARIOstar plate reader was used in endpoint mode, using emission filters 450±40 nm (80-nm bandpass) and >610 nm (longpass). The wavelengths were read sequentially for each well (well multichromatics) at room temperature (integration time: 500 ms; gain: 3000 for both channels).

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Keywords: **GPCR, NanoBRET, NanoLuc luciferase, Receptor ligand binding, TAMRA**

The PHERAstar® FS was used for the competition binding experiments and the saturation binding experiments with propranolol-BY630; endpoint mode, NanoBRET™ module (460 nm (80-nm bandpass) and >610 nm (longpass)). Both wavelengths were measured simultaneously at room temperature (integration time, 1000 ms; 460 nm gain, 2800; >610 nm gain, 3000).
The raw BRET ratio was calculated by dividing the >610-nm emission by the 460nm emission. The term "raw BRET ratio" is used, as no background ratio was subtracted.

Results & Discussion

In initial experiments, sets of HEK293 cells expressing the $ß_2$AR N-terminally-labelled with either Rluc8 or Nluc were both found to give a luminescent signal on addition of their respective substrates. The Nluc signal maximum was at 460 nm, compared to 480 nm for Rluc8 (Fig. 2). However, the relative signal strength was about 70 times greater for Nluc compared to Rluc8 (see data in Stoddart et al.[3]).

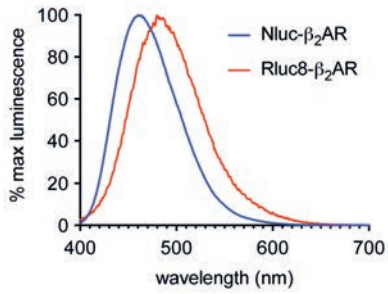

Fig. 2: Normalised emission spectra of HEK293 cells expressing $ß_2$AR N-terminally labelled with either Nluc (blue) or Rluc8 (red) using the CLARIOstar. Data previously published in Stoddart et al.[3]

Although the emission spectrum of Nluc has a maximum at 460 nm, it has appreciable intensity to ~600 nm (Fig 2). This allows the use of a range of fluorescent labels on the agonist/antagonist GPCR binding partners of interest. For HEK293 cells expressing Nluc-$ß_2$AR, the $ß_2$AR antagonist alprenolol was labelled with TAMRA (Ex 565, Em 580).

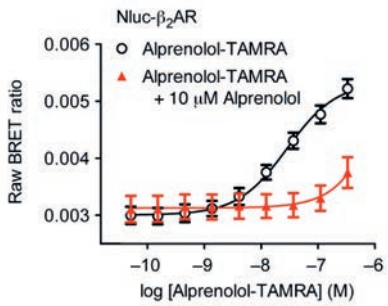

Fig. 3: Saturation binding experiment using HEK293 cells expressing Nluc-$ß_2$AR with increasing concentrations of alprenolol-TAMRA (black) and with increasing concentrations of alprenolol-TAMRA in the presence of a high concentration of unlabelled alprenolol (red). Data previously published in Stoddart et al.[3]

Specific ligand binding was observed, which could be completely prevented by competition with unlabelled alprenolol (10 µM) (Fig. 3).

A second fluorescent ligand was then assessed, propranolol-BY630. From saturation binding experiments with this labelled antagonist binding to the receptor, an equilibrium binding constant (K_D) of 18.9 ± 4.1 nM was calculated.

Most importantly, the BRET signal that was generated from the interaction of propranolol-BY630 with the receptor could be decreased by increasing concentrations of the unlabelled $ß_2$AR antagonists propranolol, ICI 118551 and CGP 12177, and the agonist isoprenaline (Fig.4). From these experiments, pKi values could be calculated from the IC_{50} values of the three antagonists propranolol, ICI 118551 and CGP 12177 (8.13 ± 0.05, 8.04 ± 0.04 and 8.32 ± 0.03 respectively).[3] Furthermore, these values were similar to those published using radioligand binding experiments,[4] highlighting the suitability of this assay for drug discovery and profiling.

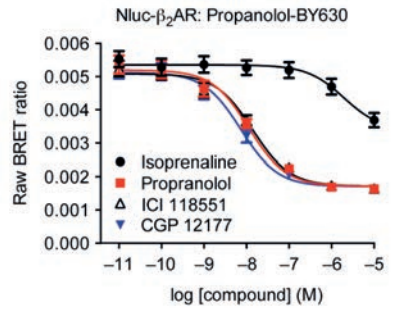

Fig. 4: Competitive binding experiments of propranolol-BY630 with increasing concentrations of known unlabelled $ß_2$AR ligands. Data previously published in Stoddart et al.[3]

Conclusion

The above study firmly establishes the NanoBRET™ assay as a viable alternative to existing methods for determination of ligand binding to GPCRs. The method will be useful in basic research as well as the pharmaceutical industry.

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HTRF® IP-One assay performed on the PHERAstar® *FS* microplate reader

Francois Degorce and Jean-Luc Tardieu
Cisbio international, France

- **HTRF® IP-One assay for Gq pathway investigation under HTS conditions**
- **Excellent performance of the PHERAstar® *FS* in terms of EC_{50} and Z´ (>0.78)**
- **Comprehensive list of GPCRs already validated with IP-One assay for agonist responses**

Introduction

HTRF® (homogeneous time-resolved fluorescence) technology, developed by Cisbio international, is used in assay development and drug screening. HTRF® is based on FRET between a Eu^{3+} cryptate (donor) and a second fluorescent label (acceptor). A new acceptor, d2, allows the introduction of a complete GPCR (G protein-coupled receptor) platform suitable for drug discovery.
Upon activation, GPCRs carry the information within the cell via two major signalling pathways: the activation of Gαs or Gαi coupled GPCRs results in a variation of the cAMP level, whereas the activation of Gq coupled GPCRs result in a transient increase of intracellular Ca^{2+} triggered by inositol (1,4,5) tri-phosphate (IP3). Cyclic AMP and IP3 therefore represent two essential secondary messengers for monitoring the activity of most GPCRs.

Concerning the Gq pathway, the precursor molecule for the signaling cascade, IP3, is an extremely instable product (turnover only a few tens of seconds) and its degradation is irreversible. IP3 induces a transient calcium release in the cell. Calcium sensing, through a very remote indicator, can allow the high throughput investigation of GPCR activity. To date, there has been no widespread use of IP assays in HTS, given its extremely challenging implementation. For lack of better options, the reference method consists of an assay which detects the accumulation of the cascade's different inositol phosphates (IP3, IP4, IP2 and IP1) after the radioactive precursor's uptake and a separation by affinity.

Recently Cisbio launched a major new kit, HTRF® IP-One, for Gq pathway investigation under HTS conditions. The IP-One kit allows the quantification of the cellular accumulation of inositol 1 phosphate (IP1).
This application note focuses on the IP1 assay which has been validated on a broad and representative selection of Gq coupled receptors using the multimode plate reader PHERAstar *FS* from BMG LABTECH. This HTRF® certified compatible reader is capable to simultaneously detect the fluorescence at two wavelengths (620 nm and 665 nm) and further signal ratioing also enables the technology to overcome interference from the medium or from compounds.

Assay Principle

The final stage of the cascade when IP1 is transformed into myo-inositol can be blocked with the use of LiCl, which enables one of the Gq pathway's essential sub-products to be stabilized (figure 1), just as IBMX action prevents cAMP degradation. In its final configuration, it brings into play a highly specific MAb coupled to europium cryptate and the IP1 conjugated to the new HTRF® acceptor, d2. This tiny proprietary molecule, with photophysical characteristics similar to those of XL665 (the reference HTRF® acceptor), improves the technology's performances markedly, particularly in terms of IC_{50} stability and of measurement dynamics.

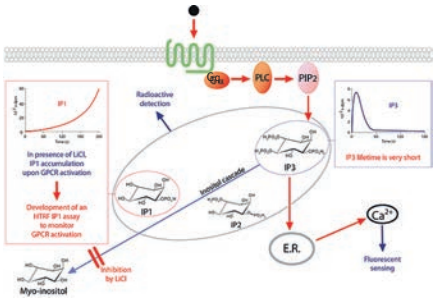
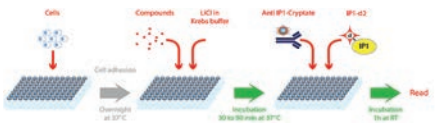


Fig. 1: Gq coupled receptor activation induces IP3 release catalysed by PLC. IP3 degradation occurs rapidly and leads ultimately to the production of IP1. Processing of IP1 into myo-inositol can be prevented by the addition of LiCl.

Materials & Methods

Cisbio's homogeneous IP-One assay can be carried out in a single microplate, into which the cells have been dispensed the day before the actual test is run. The cell stimulation conditions meet the particular characteristics of the cell line used - generally 30 minutes at 37°C. Quantification of the accumulated IP1 is obtained after dispensing the two diluted conjugates into the lysis solution.
Measurements can be taken after just one hour of incubation, and be repeated as many times as necessary without impacting final data (e.g. IC_{50}). The standard curve for the kit was run according to the package insert protocol in white 384-well plates (COSTAR cat# 3711384) with 20 µL total assay volume. The HTRF® signal was read on the PHERAstar *FS*.



Cells were kindly provided by the Institut de Genomique Fonctionnelle (IGF), Montpellier, France and Euroscreen, Gosselie, Belgium.
Cell lines (see table 1) expressing the GPCR target

Rev. 12/2005
Keywords: **CHO, GPCR, HEK293, HTRF, IP-One**

of interest were used for measuring IP1 production by stimulation of the ligand. In a 384-well format, the cell suspension was dispensed at 15,000 cells/20 µL/well. After incubation at 37°C, the culture supernatants were completely discarded. Then 10 µL of stimulation buffer containing various concentrations of ligand were added. After incubation at 37°C for 1hr, 5 µL IP1-d2 conjugate followed by 5 µL of Eu-cryptate labeled anti-IP1 antibody were added. Time-resolved fluorescence at 620 nm and 665 nm were measured with PHERAstar *FS* after incubation at 4°C overnight, and the ratios of the signals and Delta F were calculated.

Delta F % = (Standard or sample Ratio – $Ratio_{neg}$)/$Ratio_{neg}$ x 100

Results & Discussion

As shown in table 1, IP-One has already been validated on different models and targets - even if the cell lines involved were not specifically optimized for IP-One assay.

Table 1: List of GPCRs already validated with IP-One assay for agonist responses. The GPCR expression in the cell is stable (s), transient (t) or endogenous (e). HTRF® IPOne assays were performed on BMG LABTECH's plate reader.

GCPR Target	Cell Line	Agonist	EC_{50} HTRF® IP-One	EC_{50} Isotopic method
Muscarinic M1/Gq (s)	CHO-K1	Acetylcholine	71 nM	42 nM
		Carbachol	296 nM	300 nM
Vasopressin V1A/Gq (s)	CHO-K1	Vasopressin	1 nM	0.4 nM
		Vasopressin	1.6 nM	0.4 nM
Oxytocin OT/Gq (s)	CHO-K1	Oxytocin	13 nM	7 nM
Histamin H2/G16 (s)	CHO-K1	Amthamine	21 nM	16 nM
Purinergic P2Y1/Gq (s)	1321N1	2-methylthio ADP	6.8 nM	n.d.
Cholecystokinine CCK1/Gq (s)	1321N1	CCK8 sulfated	2 nM	43 nM
Chemokine CCR5/G16 (s)	CHO-K1	RANTES	76 nM	26 nM
		MIP1 alpha	48 nM	n.d.
HupCar/Gq (s)	CCL39	Calcium	2.9 mM	n.d.
Endothelin Etb/Gq (s)	CHO-K1	Endothelin 2	82 nM	83 nM
		Ala-Endothelin	70 nM	93 nM
TRH1/Gq (s)	CHO-K1	TRH	0.8 nM	n.d.
GB1+GB2/Gqi9 (t)	HEK293	GABA	980 nM	484 nM
mGluR 1/Gq (t)	HEK293	Quisqualate	113 nM	75 nM
mGluR 5/Gq (t)	HEK293	Quisqualate	13 nM	9 nM
Muscarinic M3/Gq (e)	HEK293	Acetylcholine	20 µM	n.d.
Purynergic P2Y1/Gq (e)	HEK293	UTP	2.1 µM	n.d.
		ATP	1.6 µM	n.d.

The validations also conclusively demonstrate the assay's performances in the presence of cell lines using Ga16 or Gqi9 type chimeric constructions.

The IP-One assay showed equally good performance on different cellular backgrounds, stable or transient transfected cells, and chimeric constructs. There is a strong correlation with reference methods and no cross reactivity with 50 µM of the following (phospho) inositides phosphates could be observed: Myo-inositol, PIP2, PIP3, IP2, IP3 and IP4.

In addition, the IP-One assay was applied upon the 1321N1-CCK1 cell line together with the agonist CCK8-sulfated. The experiment was performed in 96-well plates resulting in very close EC_{50} values and a very good correlation regarding the %inhibition/basal curve (figure 2). Z' calculations at an agonist concentration equal to EC_{80} resulted in Z' > 0.78.

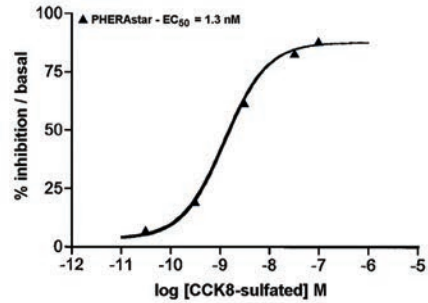


Fig. 2: HTRF® IP-One assay. The 1321N1-CCK1 cell line and CCK8-sulfated as agonist were applied.

Calcium measurement is often not sensitive enough to specifically detect the inhibition of the GPCR constitutive activities, whereas a modulation in the concentration of IP1 is perfectly able to show this.

The messenger's stability opens the possibility of the IP-One assay's application to cases where other HTS technologies fail to provide a satisfactory solution – such as in the detection of inverse agonist activities. The assay also profits from the special qualities of d2, one of the latest improvements implemented in HTRF® technology.

Conclusion

Cisbio's new IP-One assay, which is based on its proprietary HTRF® technology, is the first high throughput system that can easily detect inositol(1)phosphate (IP1), which tightly correlates with Gq-coupled activity.

All HTRF® IP-One assay results were produced on BMG LABTECH's the multimode plate reader PHERAstar *FS*. The reader showed strong results in terms of EC_{50} and Z' values.

Cisbio's HTRF® cortisol assay performed on the PHERAstar® *FS*

Jean-Luc Tardieu and Francois Degorce
Cisbio International, France

- **11 beta-hydroxysteroid dehydrogenase type 1 (11 beta-HSD1) is a key enzyme in everyday metabolism**
- **Cisbio's cortisol assay performed on the PHERAstar® *FS* to determine 11 beta-HSD1 activity**
- **EC_{50} values for cortisol and IC_{50} values for common inhibitors determined**

Introduction

Cortisol is a corticosteroid hormone present in many metabolic processes, inducing key enzymes of carbohydrate, fat and protein metabolism. Cortisol also acts as an anti-inflammatory and immuno-suppressor. One way to create this important hormone is by the reduction of cortisone by NADPH dependent 11 beta-hydroxysteroid dehydrogenase type 1 (11 beta-HSD1). This enzyme can be found in several tissues but it is mostly present in liver and fat cells.

Recently Cisbio developed an assay to determine the activity of 11 beta-HSD1 using HTRF® technology. HTRF® (Homogeneous Time-Resolved Fluorescence) is based on TR-FRET (time-resolved fluorescence resonance energy transfer), a combination of FRET chemistry and the use of fluorophores with long emission halflives (Europium, Eu^{3+}). FRET uses two fluorophores, a donor and an acceptor. Excitation of the donor by an energy source (e.g. flash lamp or laser) triggers an energy transfer to the acceptor if they are within a given proximity to each other. The acceptor in turn emits light at a given wavelength.

BMG LABTECH's PHERAstar *FS* is a multifunctional plate reader that combines rapid plate reading necessary for high throughput screening (HTS) with the enhanced performance and sensitivity needed to read small fluid volumes. The PHERAstar *FS* has been designed to read all HTS detection modes (fluorescence intensity, time-resolved fluorescence, fluorescence polarization, luminescence, AlphaScreen® and absorption) in all plate formats up to 1536 wells. To meet the HTRF® requirements the PHERAstar *FS* uses a unique HTRF®-specific optical module that can measure two emission signals simultaneously.

Assay Principle

The cortisol assay is a monoclonal antibody based competitive assay (Figure 1). It is run in two steps. After the dehydrogenase reaction is finished (stimulation step), anti-cortisol cryptate (donor) and d2 labeled cortisol (acceptor) are added to the reaction mix. The anti-cortisol cryptate and the d2 labeled cortisol will bind to each other leading to a high HTRF® signal. Cortisol built during the enzymatic reaction will compete with d2-labeled cortisol for the binding to the cryptate conjugate, resulting in a loss in HTRF® signal (detection step).

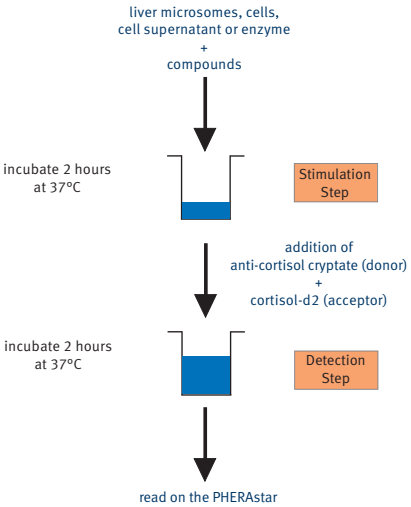


Fig. 1: Principle of Cisbio's cortisol assay.

Materials & Methods

Instruments

BMG LABTECH's PHERAstar *FS*, Offenburg, Germany
HTRF® optical module (excitation: 337 nm, emission A: 665 nm and emission B: 620 nm), Offenburg, Germany
Cisbio's HTRF® Cortisol assay Bagnols, France

Cortisol standard curve

10 µL of cortisol standards (serial dilution) and 5 µL of each HTRF® conjugate (anti-cortisol cryptate and cortisol-d2) were dispensed into the wells of a black 384 small volume microplate from Greiner. The plates were measured using the HTRF® module after both two hours and 16 hours of incubation.

Biochemical assay

Inhibitor dose response curves 2 µL of 11beta-HSD1 microsomal preparation (0.1 mg/mL) in Tris 20 mM EDTA 5 mM buffer (pH = 6), 6 µL of Tris 20 mM, EDTA 5 mM buffer (pH = 6) containing cortisone 266 nM and NADPH 333 µM and 2 µL of inhibitor (carbenoxolone and glycyrrhetinic acid) at different concentrations in Tris 20 mM EDTA 5 mM buffer were dispensed into the wells. After 2 hours of incubation at 37°C, 5 µL of each HTRF® conjugate (anti-cortisol cryptate and cortisol-d2) were added. The plates were incubated for another 2 hours at room temperature before reading on the PHERAstar *FS*.

Rev. 03/2007
Keywords: **Cortisol, Hormone, HTRF, Inhibitor dose response, Steroids**

Data analysis

The signal is expressed in DeltaF in % (DF%)

$$DF\% = \frac{Ratio_{pos.\ control} - Ratio_{neg.\ control}}{Ratio_{neg.\ control}} \times 100$$

where Ratio = (Signal at 665nm / Signal at 620nm) x 10^4 and pos.control = positive control and neg.control = negative control.

The ratio is automatically calculated by the PHERAstar *FS* MARS data analysis software.

Results & Discussion

Figure 2 shows cortisol titration curves at different incubation times.

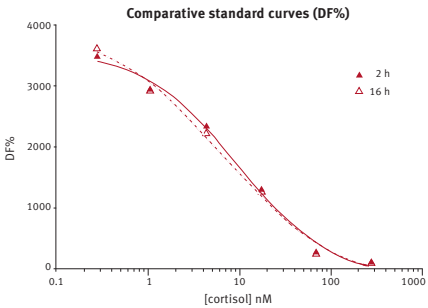


Fig. 2: Cortisol titration curves recorded on the PHERAstar *FS* after 2 hours and 16 hours incubation.

This data demonstrates that with increasing cortisol concentration the anti-cortisol cryptate is displaced proportionally resulting in a decreasing signal curve.

Table 1: EC_{50} values of cortisol standard curve after different incubation times.

Inhibitor	IC_{50} µM
Carbenoxolone	1.03
Glycyrrhetinic acid	1.17

Conclusion

The cortisol assay allows fast and efficient determination of cortisol in complex samples such as serum and whole cells. Screening for both active 11 beta-hydroxysteroid dehydrogenase type 1 and its inhibitors is also simple and effective using this homogeneous assay.

The PHERAstar *FS* in combination with the optimized HTRF® optical module is the ideal tool to run HTRF® assays. The PHERAstar *FS'* optical design provides for outstanding sensitivity and accuracy in fluorescence and luminescence assays; moreover, the dual simultaneous measurement minimizes the read time for assays.

PHERAstar® *FSX*
PHERAstar® *FS*

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A DELFIA® time-resolved fluorescence cell-mediated cytotoxicity assay performed on the PHERAstar® *FS*

N.M. Patterson, T. Cunningham, X. Jiang and D.J. Shapiro
University of Illinois, Urbana, IL

- **A europium time-resolved fluorescence cytotoxicity assay in a 96 well format is analyzed by an HTS reader**
- **The data herein shows that estrogen dramatically reduces cell death induced by NK92 natural killer cells**
- **This time-resolved fluorescence assay is shown to be an alternative to the radioactive chromium release assay**

Introduction

Estrogens are steroid hormones that act via estrogen receptors to exert both intranuclear and extranuclear effects in human cells. By this way, estrogens promote different cellular processes like proliferation and metastases in human cancers; in addition, estrogen has been shown to play a role in tumors, developing the ability to block immunosurveillance. Immunosurveillance results primarily from apoptosis of neoplastic cells induced by natural killer (NK) cells and cytotoxic T lymphocytes (CTLs). These cytolytic lymphocytes (CLs) use perforin and granzyme-containing granules. Granzyme B (GrB), is thought to play a leading role in granzyme-mediated cytotoxicity to induce lysis of target cells.

It was shown that in breast cancer cells, increasing concentration of estrogens induce increasing levels of granzyme B inhibitor and SerpinB9/Proteinase Inhibitor 9 (PI-9), while progressively blocking cell death induced by NK92 natural killer cells. Several assays are available to monitor apoptosis and other forms of cell death. These assays focus on changes that occur during apoptosis – the annexin V and propidium iodide assays are based on changes in membrane properties and permeability, and the TUNEL assay is based on apoptosis-related DNA fragmentation.

These and other assays are extremely good in evaluating apoptosis in a homogenous cell population, but are less useful when evaluating apoptosis in only one type of cell when a mixture of cells is present. One common example of cell death in a mixed cell population occurs in the process termed immunosurveillance in which cells of the immune system, such as cytotoxic T lymphocytes (CTLs) and Natural Killer (NK) cells, induce apoptosis of target cells. Assaying apoptosis in mixed cell populations usually requires the cells of interest to be pre-loaded with a reagent, and the loss of membrane integrity that occurs during cell death results in the reagent being released. The most widely used assay is to pre-load cells with radio-labeled chromium. However, 51Cr is an unstable photon emitter and requires costly and cumbersome lead shielding for safe use.

Here the PHERAstar® *FS* HTS microplate reader is used in a time-resolved fluorescence assay to study the loss of membrane integrity. In this assay, target cells are loaded with fluorescence enhancing ligand, BATDA, which penetrates the cell membrane quickly and is hydrolyzed to form a hydrophilic ligand (TDA) that is released from cells only after cytolysis (Figure 1).

In the presence of a solution containing Eu^{3+}, the released TDA forms a highly fluorescent and stable chelate (EuTDA), whose levels are measured. It is shown that this assay produces results that are similar to the classical chromium release assay.

Assay Principle

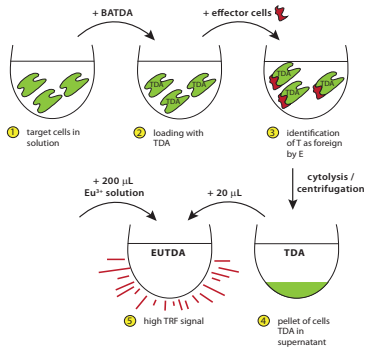

Fig. 1: Principle of the DELFIA® cytotoxicity assay.

BATDA will pass the cell membrane of the target cells. Intracellular esterases cleave ester bondages leading to a hydrophilic ligand that cannot pass the membrane (TDA). Effector cells recognize their target cells as foreign and the cytolysis process is initiated. After cytolysis the TDA is released and is found to be in the supernatant after centrifugation. A 20 µl aliquot of supernatant is added to 200 µl of Eu^{3+} solution resulting in a high time-resolved fluorescent signal that is measured on the PHERAstar *FS*.

Materials & Methods

- 96-well V-bottom black microplates
- DELFIA® EuTDA Cytotoxicity Reagents including BATDA Reagent, Lysis buffer and Europium Solution, PerkinElmer
- PHERAstar *FS*, BMG LABTECH

The cell mediated cytotoxicity assay was carried out following the manufacturer's protocol with a few modifications. Target cells (MCF-7, human breast cancer cells) were incubated at 10^6 cells/mL in phenolred free MEM plus 5% CD-calf serum with BATDA for 10 min at room temperature, followed by 4 washes.

The target cells were then mixed with effector cells at different effector cells/target cells (E/T) ratios. Cytotoxicity assays were carried out in 96-well V-bottom plates containing 10,000 target cells in a total volume of 200 µL/well. The plates were subjected to centrifugation for 30 seconds at 1,000 rpm to facilitate contact between target and effector cells.

After incubating for 2 hours, supernatant from each reaction (20 µl) were added to 200 µl of the Eu^{3+} solution.

Rev. 06/2009
Keywords: **Cell death, Cytotoxicity, Delfia, Estrogen, NK92 natural killer cells**

Following agitation of the plates for 5 min, time-resolved fluorescence was measured by using an application specific DELFIA® module with an excitation wavelength of 337 nm and an emission wavelength of 615 nm using a PHERAstar *FS* microplate reader.

Calculation

Percent specific lysis was calculated as:

$$100 \times \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}}$$

Spontaneous release

Target cells are incubated with medium instead of effector cells.

Maximum release

Target cells are incubated with lysis buffer instead of effector cells.

Results & Discussion

Using the described time resolved fluorescence assay that detects release of a fluorescent substrate from cells that lose membrane integrity, estrogen treatment for 24 hours dramatically reduced cell death induced by NK92 cells (Fig. 2).

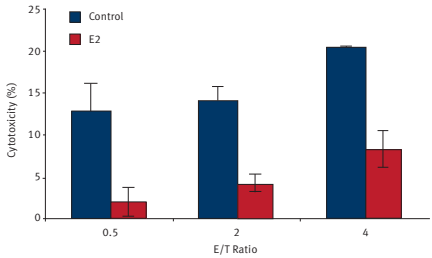

Fig. 2: Estrogen (E2) protects MCF-7 cells against cytolysis induced by NK92 cells. MCF-7 target (T) cells were treated with ethanol vehicle (filled bars) or 10 nM E2 (open bars) for 24 hours, followed by incubation with NK92 effector (E) cells at different E/T ratios.

Proteinase Inhibitor 9 (PI-9) inhibits granzyme B and NK cells use the granzyme pathway to kill target cells. However, the diverse effects of estrogens raise the possibility that other actions of estrogen might be responsible for blocking NK cell-induced cytotoxicity. To evaluate the role of PI-9 in estrogen-mediated protection against NK cell induced cytotoxicity, we used RNAi to knock down PI-9 expression. Knockdown of PI-9 with PI-9-specific siRNA abolished the ability of estrogen to block NK92 cell-induced killing (Fig. 3). These data demonstrate that estrogen's ability to block NK cell mediated cytolysis of MCF-7 cells derives from its ability to induce PI-9.

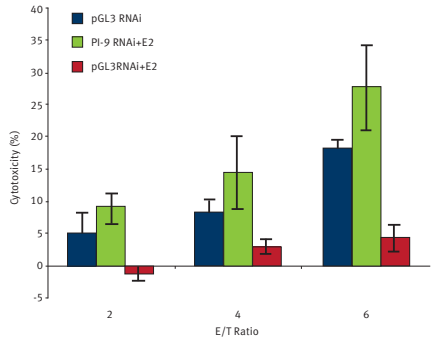

Fig. 3: RNAi knockdown of PI-9 blocks estrogen protection against NK cell mediated cytotoxicity. MCF-7 cells were transfected with the control pGL3 luciferase siRNA, or with the PI-9 siRNA. After 24 hours, ethanol vehicle or estrogen (E2) was added and the cells were maintained for an additional 24 hours and incubated with the indicated ratios of effector NK92 cells to MCF-7 target cells and assayed for cytoxicity using the time resolved fluorescence assay.

Conclusion

With the DELFIA® cytotoxicity assay measured on the PHERAstar *FS* it is possible to investigate NK cell mediated cytolysis. The data indicate a clear relationship between increasing estrogen and reduced cell lysis. Furthermore it was shown that estrogen protects the MCF-7 cells by utilizing Proteinase inhibitor-9.

GPCR activation is measured using Cisbio's cAMP and IP1 HTRF® HTplex™ cell-based assay

Laurence Jacquemart[1] and E.J. Dell[2]
[1] Cisbio Bioassays [2] BMG LABTECH

- **Lumi4-Tb™ HTRF® technology is used to measure both cAMP via a green readout (520 nm) and IP1 via a red readout (665 nm)**
- **Activation of the vasopressin-2 receptor was measured in CHO cells**

Introduction

GPCRs carry information within cells via two major signaling pathways: regulation of cAMP levels and increases in intracellular Ca^{2+} triggered by inositol 1, 4, 5- triphosphate (IP3). These signaling pathways are activated by the specific G protein associated with the receptor. Activation of a Gs or Gi coupled receptor results in the increase or decrease of cAMP levels, respectively. While activation of a Gq coupled receptor activates phospholipase C (PLC) and triggers the inositol phosphate (IP) cascade (Figure 1).

Cisbio Bioassays has developed assay kits capable of monitoring the activation of Gs, Gi and Gq coupled receptors, using their new generation Lumi4-Tb™ TR-FRET Cryptate. This chemistry allows for the detection of two events in one well using two different acceptors, a green dye (λ = 520 nm) and a red dye (λ = 665 nm). This same chemistry is used in Cisbio's Tag-lite® technology.

IP-One and cAMP experiments were performed on a HTS microplate reader from BMG LABTECH using Simultaneous Dual Emission detection. With this unique feature, the plate is read only once for dual emission assays, thereby decreasing time and variability.

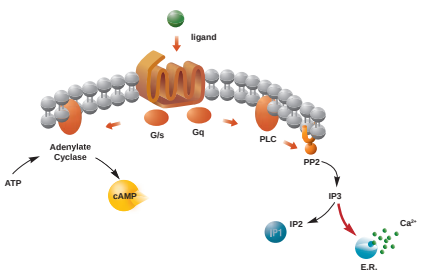

Fig. 1: The HTplex™ Assay from Cisbio measures GPCR activation via two second messenger responses, cAMP and IP1, in one experiment.

Assay Principle

The IP-One and cAMP HTplex™ assay is a competitive immunoassay that uses two antibodies labelled with Lumi4-Tb™ (anti-cAMP Cryptate and anti-IP1 Cryptate as donors) and two acceptors (cAMP-green dye and IP1-red dye). In the inactivate state, a high TR-FRET signal is seen for both the red and green emissions. As cAMP or IP1 is produced upon GPCR activation, the tracer green-cAMP or the tracer red-IP1 will be uncoupled from the Tb-cryptate antibody, thereby causing a decrease in TR-FRET signal. Specific emission signals are inversely proportional to the concentration of cAMP and IP1 in a standard or in a cell lysate.

Materials & Methods

- IP-One and cAMP reagents from Cisbio Bioassays
- White 384-well format microplate, Greiner
- Multidetection microplate reader from BMG LABTECH

Cells preparation

CHO-V2R cells (stable transfection with the vasopressin-receptor) are cultivated in F12 medium then diluted to obtain a concentration of 1,000,000 cells/mL (viability: 96.3%). Then 30 µL are distributed in each well (giving 30,000 cells/well). The plate is incubated at 37°C overnight. The cell supernatant is aspirated (the cells collapse to the well bottom) and immediately replaced with 10 µL of stimulation buffer.

For standard curve: add 20 µL of diluted standards, 5 µL cAMP-green dye/IP1-red dye and 5 µL cAMP-Cryptate/ IP1-Cryptate, then incubate for 1 hr at RT. For vasopressin dose-response: add 10 µL stimulation buffer, 10 µL vasopressin (14 dilutions: from 0 to 10^{-4} M), 1 hr stimulation at 37°C. Then add 5 µL cAMP-green dye/ IP1-red dye, 5 µL cAMP-Cryptate/ IP1-Cryptate and incubate for 1 hr. For Z' calculation: add 10 µL stimulation buffer (basal) or 10 µL vasopressin 10 µM, 1 hr stimulation at 37°C. Then add 5 µL cAMP-green dye/IP1-red dye, 5 µL cAMP-Cryptate/IP1-Cryptate and incubate for 1 hr.

Instrument settings

Specific HTRF compatible filter sets/optic modules, one for red and one for green.Integration start = 60 µs, integration time = 400 µs. Flash number = 300 (if speed is important, 100 flashes can be used). Plate is read twice. In case the instrument is equipped with a UV laser, it is recommended to use 7 flashes.

Data Reduction

Cisbio has a patented ratiometric measurement that uses both the emission wavelength of the donor and acceptor (patent US 5,527,684* and foreign equivalents) to correct for well-to-well variability and signal quenching. Emissions at 620 nm (donor) are used as an internal reference while emissions at 665 nm and 520 nm (acceptor) are used as an indicator of the biological reaction being assessed. Ratios of fluorescence intensities 665/620 and 520/620 (acceptor/donor) are calculated in order to detect each single interaction.

Rev. 10/2010
Keywords: **cAMP, GPCR, HTPlex, HTRF, IP1, Simultaneous Dual Emission SDE**

Results & Discussion

Figure 2 shows the calibration curve using standards for cAMP and IP1. Both curves give the expected EC_{50} values.

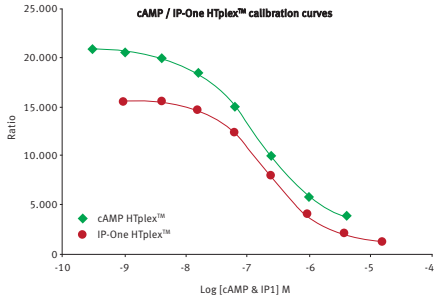

Fig. 2: Calibration curves for the HTplex™ assay using standards for cAMP (green) and IP-One (red).

This figure shows the occurrence of two signaling events - an initial cAMP (Gs) response at lower concentrations of vasopressin presumably through activation of endogenous V1 receptors; and a delayed IP3 (Gq) response at higher concentrations through activation of the transfected V2 receptor (as measured by IP1 accumulation).

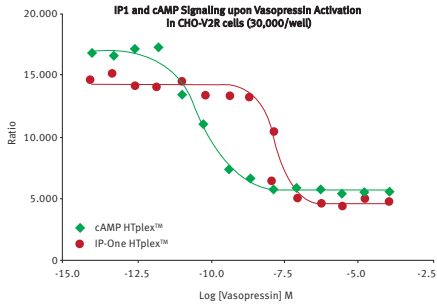

Fig. 3: GPCR signaling in CHO-V2R cells was measured via cAMP (green) and IP1 (red) responses upon a dose dependent activation of vasopressin.

In Figure 3, both cAMP and IP1 were measured in CHO-V2R cells upon activation with vasopressin in a dose response manner.
Lastly, the robustness of the assay as measured by the Z prime calculation (Figure 4) shows that both signals are well above the reliability range for an HTS assay, giving Z' values > 0.75.

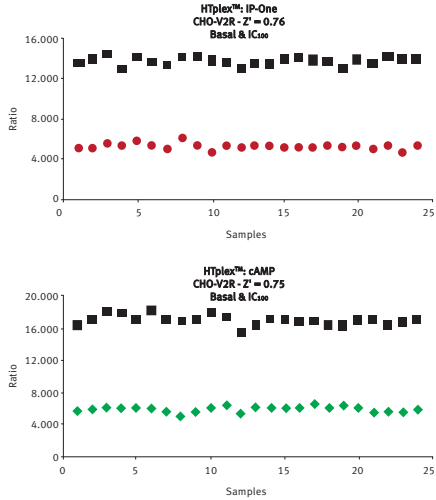

Fig. 4: Z prime calculations for IP1 (top graph, red) and cAMP (bottom graph, green) using 0 and 10 µM of vasopressin.

Conclusion

The HTplex™ assay from Cisbio can evaluate two different GPCR signaling pathways, Gαi/s and Gαq, through the measurement of their second messenger responses, cAMP and IP3 (via IP1), respectively. Herein, the HTplex™ assay was used to evaluate the dose response effect of vasopressin on CHO-V2R cells. As measured, there is an initial cAMP response at lower concentrations and a latent IP1 accumulation at higher concentrations. The concept of this application note can be extended to Cisbio's complimentary HTRF® chemistry, Tag-lite®, which uses the same Lumi4-Tb™ cryptate and the same red and green acceptors.

Assessment of extracellular acidification using a fluorescence-based assay

James Hynes[1], Conn Carey[1], Sinead Kriwan[1] and Catherine Wark[2]
[1] Luxcel Biosciences Ltd. [2] BMG LABTECH

- **Time-Resolved Fluorescence assay to measure Extracellular Acidification**
- **Measures the conversion of Pyruvate to Lactic Acid**
- **Convenient measure of glycolytic flux**

Introduction

Metabolic perturbations play a critical role in a variety of disease states and toxicities. Knowledge of the interplay between the two main cellular ATP generating pathways; glycolysis and oxidative phosphorylation, is therefore particularly informative when examining such perturbations. Here we describe a time resolved fluorescence-based assay for the assessment of Extracellular Acidification which provides data on the rate of conversion of pyruvate to lactic acid and is therefore a convenient measure of glycolytic activity. Such assays are particularly informative when assessing alterations in glucose metabolism, detecting glycolytic inhibition and as a confirmatory analysis in the identification of mitochondrial dysfunction. The following protocol outlines how such measurements can be carried out on standard microtitre plates using the fluorescent pH-sensitive probe, pH-Xtra and ratiometric time resolved fluorescence detection. This approach overcomes the calibration and biocompatibility issues associated with some existing probes thereby allowing conventional cell culturing and assay procedures whilst also facilitating accurate quantitative analysis. In addition, spectral compatibility with MitoXpress®, the Luxcel oxygen sensitive probe, facilitates a multi-plexed measurement approach providing a comprehensive metabolic assessment of test cells.

Materials & Methods

- Clear 96 well plates from Costar
- pH-Xtra probe from Luxcel Biosciences, Ireland
- Microplate reader from BMG LABTECH

Plate Preparation and Reading

Warm instrument to measurement temperature (typically 30°C). Prepare kinetic measurement protocol to read the plate at 2-4 min intervals over 1-2 h period using the recommended ratiometric measurement parameters (more detail in the "Measurement" section). Adherent cells were either (A) plated at the indicated concentration in L15 medium and cultured in CO_2-free conditions, 95% humidity at 37°C overnight or (B) cultured overnight in a standard CO_2 incubator and then maintained in CO_2-free conditions for 2.5 h prior to the measurement. Reconstitute pH-Xtra probe in 1 mL of Millipore water. Warm to measurement temperature. Wash cells with measurement buffer being careful not to dislodge cells from the base of the wells. Add 150 µL of the pre-warmed measurement buffer to each well and place the plate on a plate heater equilibrated to 30°C. Using a repeater pipette, add 10 µL pH-Xtra probe to each well. Follow with drug addition if applicable. Oil can be added if bulk acidification data is required.

Measurement and Data Analysis

Insert the microplate into the microplate reader. Glycolytic activity is measured using the BMG LABTECH scripting function. pH-Xtra probe is measured using dual delay, time-resolved measurements. Optimal filter wavelengths are 340 TR L for excitation and TR 615 for emission. Delay times of 100 and 300 µs are used, both with a measurement window of 30 µs respectively. Plate preparation time should be kept to a minimum. When the measurement cycle is completed, remove the plate from the instrument and save the measured data to a file. These dual intensity measurements are used to calculate emission lifetime using the following function, τ=t2-t1/ Ln (D2/D1) [t=delay time, D=measured intensity value]. Scripts and protocols can be obtained through your local BMG LABTECH representative and MARS templates are available to facilitate data analysis.

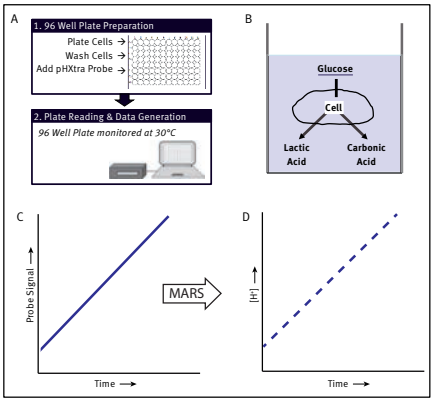

Fig. 1: Summary of pH-Xtra Assay showing a general schematic of assay preparation (A). Cellular metabolic processes (B) cause an acidification of the media resulting in an increase in probe signal (C). If necessary, this signal can be conveniently converted into a H^+ scale using a MARS template (D).

Results & Discussion

Monitoring Cellular Respiration

The ability of the pH–Xtra assay to assess cell respiration is illustrated in Fig. 2. A typical data read out is presented in Fig 2A showing the parallel analysis of 96 individual samples. A serial dilution of HepG2 is presented in Fig 2B with increasing cell numbers causing an increased rate of acidification. This is seen

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Keywords: **Acidification, Glycolytic flux, Lactic acid, Luxcel, pH, Respiration**

as in increased rate of signal change. Profiles are highly reproducible showing %CV values of the order of <5%.

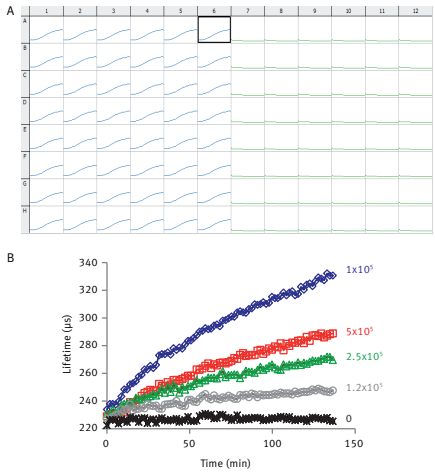

Fig. 2: **A)** 96 well plate based analysis of extracellular acidification measured on a BMG LABTECH microplate reader, cell profiles on left, control profiles on right). **B)** Acidification profiles for HepG2 cultured in L15 medium at the indicated seeding concentration (cells/well).

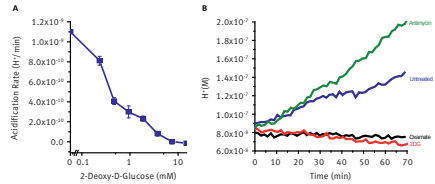

Fig. 3: **A)** Effect of increasing concentrations of 2DG on measured HepG2 acidification. Data are mean ±SD, n=4. **B)** Acidification profiles of HepG2 cells illustrating the effect of compound treatment.

Monitoring Perturbed Metabolism

Treatment with 2DG results in reduced glucose uptake due to competitive inhibition of glucose transport. Treated cells show dramatic, immediate and dose dependant decreases in rates of extracellular acidification (Fig 3A). Mitochondrial toxins such as electron transport chain inhibitors prevent or restrict aerobic ATP generation. In many cell systems this leads to an increase in glycolytic flux to supply cellular ATP. This increased acidification can therefore be used as a confirmatory parameter when investigating drug-induced mitochondrial dysfunction. These rates are typically compared to untreated cells to determine the direction and magnitude of the effect observed. Such response can be seen in Fig 3B where treatment with Sodium Oxamate and 2DG cause the expected decreases in extracellular acidification while treatment with the Electron Transport Chain inhibitor Antimycin causes a significant increase in acidification in an attempt to maintain cellular ATP supply. This assay can be multiplexed with the Luxcel MitoXpress® probe to assess both Extracellular Acidification and Oxygen Consumption on the same plate.

Conclusion

- Analysis of cellular glycolytic flux is possible on standard 96 well plates by measuring rates of extracellular acidification.
- The ratiometric measurement approach facilitated by the pHXtra probe provides a robust assay readout.
- Such measurements allow convenient cellular metabolic characterisations.
- When assessed alongside an analysis of oxygen consumption a detailed analysis of mitochondrial function is possible
- Data analysis is greatly simplified using MARS data analysis templates.

Supplemental Information

1. When culturing cells in a standard 5% CO_2 environment the polystyrene body of a standard microtitre plate absorbs CO_2. If a plate is moved immediately from 5% CO_2 to the measurement chamber this CO_2 will diffuse into the sample causing significant acidification. To circumvent this, cells may be cultured in CO_2-free conditions in specially formulated media such as L-15, or cultured in standard media and moved to a CO_2-free environment 2h prior to measurement.
2. Measurement Buffer 1: 1mM K-phosphate, 20mM Glucose, 0.07M NaCl and 0.05M KCl, 0.8mM, $MgSO_4$, 2.4mM $CaCl_2$, pH 7.4. Measurement Buffer DMEM Base, 1.85g/l NaCl, 10ml 100x GlutaMax, 10mL 100mM Sodium Pyruvate, 15 mg Phenol Red, 25mM glucose.
3. Acidification in unsealed samples is due almost entirely to lactate conversion while, in sealed samples there is a strong contribution from carbonic acid due to trapped CO_2 reflecting Krebs cycle activity.

HTS instrument discovers low affinity inhibitors of the Inositol Phosphate (IP) signaling pathway

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[1]BMG LABTECH GmbH [2]Cisbio Bioassays

- **Several low affinity drug 'hits' were only found by the PMT-based reader and not the CCD-based reader**
- **PHERAstar® *FS* performs more than twice as fast as the CCD-based reader**
- **PHERAstar *FS* shows superior assay quality parameters such as Z', DeltaF% and assay window**

Introduction

G-protein coupled receptors (GPCRs) are transmembrane proteins which play a key role in the signal transduction of extracellular stimuli. GPCRs are associated with a complex assembly of intracellular proteins regulating a large variety of downstream effectors. The production of inositol 1,4,5-triphosphate (IP3) is one such second messenger, which is produced in response to the activation of Gq-coupled receptors. However, IP3's very short half-life make its assessment too challenging for drug screening assays and the monitoring of calcium release, triggered by IP3, has been extensively used as a downstream readout of this signaling pathway.
An alternate way to monitor IP3 is to measure the accumulation of inositol monophosphate (IP1), which is a downstream metabolite of IP3.

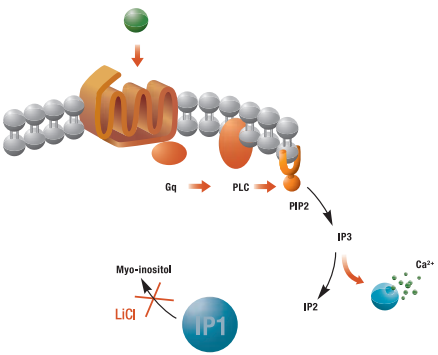

Fig. 1: Biosynthesis of IP1.

Monoclonal antibodies raised against IP1 lead to the optimization of a homogeneous time-resolved fluorescence assay (HTRF®), taking advantage on the fact that IP1 is stable and accumulates in cells. The HTRF® IP-One assay has been compared to existing methods and has been shown to lead to similar compound potency data. Moreover, the end point accumulation of IP1 allows for the discrimination of slow acting compounds that remain unseen by calcium sensing. The HTRF® IP-One assay also allows for the characterization of inverse agonists by the quantification of constitutively active GPCRs, which is impossible via measurement of calcium release. Lastly, HTRF® IP-One detection confers superior assay robustness and much lower false positive rates compared to calcium detection. The IP-One assay developed by Cisbio Bioassays uses their proprietary HTRF® technology (Figure 2).

Assay Principle

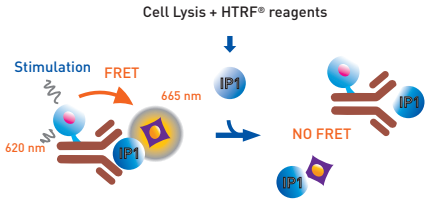

Fig. 2: IP-One HTRF® Assay Principle.

The assay uses a monoclonal antibody that specifically recognizes IP1 and it is based on a competition format in which the intracellular accumulation of IP1 inhibits the fluorescence resonance energy transfer (FRET) signal between the HTRF® donor and acceptor. An IP1 calibration curve can estimate the IP1 concentration accumulated in cells as a function of the compound concentration.

Materials & Methods

- Next generation PMT based HTS microplate reader, PHERAstar *FS*
- HTS CCD-based microplate imager from a different vendor

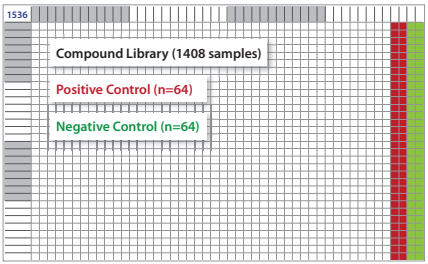

Fig. 3: 1536-well microplate layout.

Black Greiner 1536-well microplates were used with an assay volume of 5 µL. As shown in Figure 3, 1408 different compounds were pipetted into the first 44 columns of a microplate, with positive (POS, n=64) and negative (NEG, n=64) controls in the last 4 columns.
The PHERAstar *FS* is equipped with a high power pulsed nitrogen laser emitting at 337 nm, as well as a dedicated Simultaneous Dual Emission (SDE) direct photon counting time-resolved fluorescence mode. When exciting the terbium (Tb) donor molecule, the

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Keywords: **1536-well, CCD, GPCR, HTRF, Screening, Simultaneous Dual Emission SDE**

laser is superior to a broadband xenon flash lamp. The laser's energy emission takes advantage of the higher molecular extinction coefficient of the terbium cryptate peaking around 337 nm, compared to europium (Eu).

Results & Discussion

Results were evaluated by the HTRF® ratio of the two emission wavelengths (Em 665 nm / Em 620 nm) and hits are shown as peaks in a surface graph representing the entire 1536 well microplate.

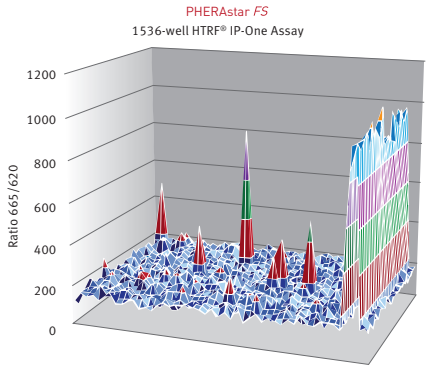

Fig. 4: HTRF® ratios obtained for the IP-One assay with the PHERAstar *FS*.

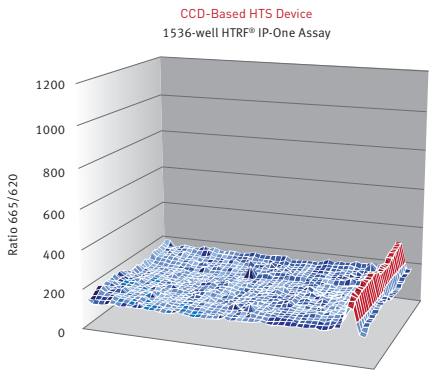

Fig. 5: HTRF® ratios obtained for the IP-One assay with a CCD-based HTS Device.

Table 1: Speed and assay quality are compared between the PHERAstar *FS* and the CCD camera based HTS reader.

	PHERAstar *FS*	CCD based HTS reader
Read Times (1536)	53 sec	2:12 min
Assay Window	6:1	2:1
Delta F%	490	76
Z' Value	0.70	0.24

Conclusion

The IP-One HTRF® assay from Cisbio was performed on two different HTS microplate readers with different detection technologies. Low affinity compounds which were not discovered with a leading HTS CCD camera based imaging microplate reader were readily resolved with the PMT based PHERAstar *FS*. This next-generation HTS reader, the PHERAstar *FS* from BMG LABTECH, represents a new choice for HTS screening assays.

PHERAstar® *FSX*
PHERAstar® *FS*

Real-time monitoring of intracellular oxygen using MitoXpress®-Intra

James Hynes[1], Conn Carey[1], and Catherine Wark[2]
[1] Luxcel Biosciences Ltd. [2] BMG LABTECH

- **Convenient, real-time monitoring of intracellular oxygen within a cell monolayer**
- **Membrane permeable, ultra-sensitive, probe increases as oxygen levels are depleted within the cell**
- **Easily assess transient changes in metabolic activity, as well as hypoxia, which extracellular probes cannot do**

Introduction

Molecular oxygen is a key substrate of all aerobic organisms and the terminal acceptor of the electron transport chain (ETC). This highly informative marker of cellular metabolism and mitochondrial function is closely regulated by the ATP/ADP ratio, the levels of available oxygen and the action of signalling molecules such as NO and Ca^{2+}. Analysis of molecular oxygen facilitates the elucidation of critical biochemical pathways; including cell survival and death, mitochondrial function, toxicological impact of compounds and metabolic alterations caused by various stimuli or disease states. To date, routine analysis of intracellular molecular oxygen has been limited by the lack of suitable means of sensing oxygen within the cell monolayer.

Assay Principle

MitoXpress®-Intra is an oxygen-sensitive probe developed for the intracellular analysis of molecular oxygen using plate-based time-resolved fluorometry. Measurement is based on the ability of O_2 to quench the emission of a probe which is taken up by endocytosis. As cells respire, the concentration of oxygen within the cell monolayer is depleted and this depletion is seen as an increase in probe signal, expressed as probe lifetime, following ratiometric measurement of time-resolved phospho-rescence intensity. This allows real-time information on the concentration of molecular oxygen within the cell monolayer to be generated across multiple samples without a requirement for specialised imaging equipment. The ability to monitor such fluctuations is particularly informative as it allows the assessment of transient changes in metabolic activity and provides access to a critical parameter in the study of hypoxia which is beyond the capacity of extracellular sensing methodologies.

Materials & Methods

- MitoXpress®-Intra ($NanO_2$) reagent from Luxcel Biosciences
- BMG LABTECH microplate reader equipped with Atmospheric Control Unit (ACU)

Probe preparation and cell loading

Cells are grown to full confluence in a suitable medium supplemented with foetal bovine serum, L-glutamine and Penicillin/ Streptomycin on cell culture treated 96 well plates. Replace seeding medium with 100 µl of normal growth media (10% FBS) containing MitoXpress®-Intra probe at 10µg/ml and return to culture overnight. Prior to measurement, replace media with 150 µl of pre-warmed media.

Measurement

Intracellular oxygen in samples is measured using the BMG LABTECH microplate reader and the scripting function. Optimal filter wavelengths are 340 TR L for excitation and EM 655-50 for emission in Omega readers and EM 645-20 for CLARIOstar. Phosphorescent intensities are measured at delay times of 30 µs and 70 µs (with 30 µs window time) with the ratio of these intensities subsequently converted to phosphorescent lifetimes as shown in Figure 1. These conversions are performed with the help of the MARS data anaylsis software.

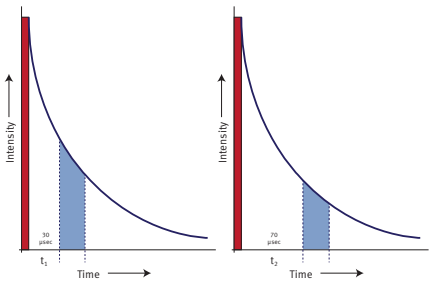

Fig. 1: Measurement principle for calculation of phosphorescence lifetime from ratiometric time-resolved phosphorescence intensity.

Results & Discussion

Typical Data Output

When analysing a fully confluent monolayer, ETC activity results in measurement usually beginning at oxygen concentrations below air saturated levels. Reduced ETC activity results in an increase in intra-cellular oxygen concentration resulting in a decrease in probe signal while increased ETC activity results in a decrease in intracellular oxygen concentration resulting in an increase in probe signal (Fig 2). Increases may be prolonged or more transient, depending on the processes involved and the measurement conditions.

Rev. 03/2012
Keywords: **ACU Atmospheric Control Unit, Life times, Luxcel, MitoXpress, Oxygen**

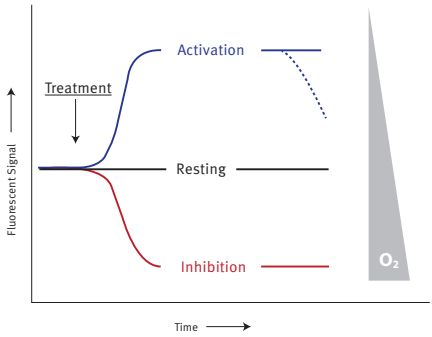

Fig. 2: Diagrammatic representation of MitoXpress®-Intra probe profiles in response to drug treatment.

Probe Response

Measurement of phosphorescent lifetime of the MitoXpress®-Intra probe facilitates measurement of the basal level of intracellular oxygen within the cell monolayer. The response of the probe is demonstrated in Antimycin treated HepG2 cells measured on a microplate reader equipped with an atmospheric control unit (BMG LABTECH). This instrument allows the ambient O_2 concentration to be reduced stepwise. The time taken for the non-respiring monolayer to equilibrate to this new O_2 concentration can be observed (Fig. 3) with the new steady state probe signal reflecting this new concentration. Such calibrations also allow measured lifetime values to be automatically transposed into O_2 concentrations, thereby allowing probe response to be viewed in this scale.

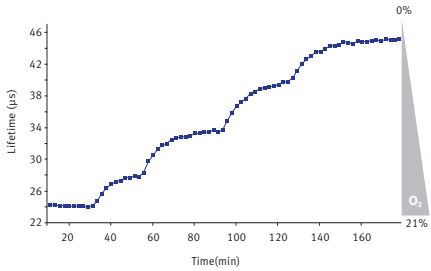

Fig. 3: Kinetic probe trace showing the response of the cell monolayer to decreasing ambient oxygen.

Assay Performance & Cell Responses

Figure 4A shows the effect of HepG2 cells treated with an electron transport chain inhibitor (Antimycin) and uncoupler (FCCP). The magnitude, consistency of monolayer (de)oxygenation is monitored. Profiles are seen to be highly consistent with uncoupling causing enhanced oxygen consumption seen by an immediate decrease in intracellular oxygen resulting in an increase in probe lifetime while inhibition causes the reverse. Increasing the FCCP concentration increases the speed of the deoxygenation while increasing the cell number increases the depth of the deoxygenation (data not shown). Treatment of HCT116 cells with the RyR1 agonist ryaniodine also causes a transient de-oxygenation of the monolayer via a calcium mediated increase in metabolic rate (Fig. 4B), a response not readily apparent using extracellular analysis.

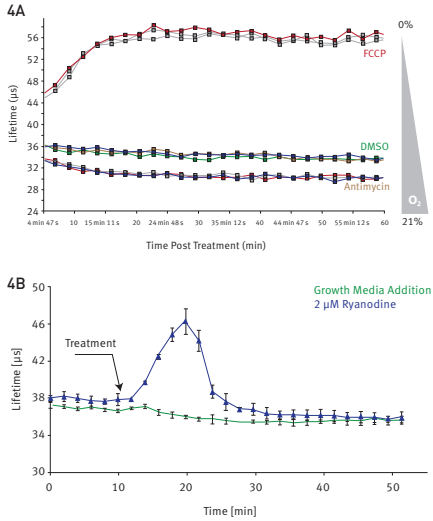

Fig. 4: Consistency of response & inhibition and uncoupling of ETC activity (A) and kinetic analysis of metabolic responses agonist treatment (B).

Conclusion

MitoXpress®-Intra allows the convenient monitoring of intracellular oxygen on a conventional microtitre plate format. The probe is 'self loading' and provides excellent signal to blank performance. This allows the investigation of a variety of parameters currently beyond the capabilities of existing probe technologies.

Atmospheric Control Unit (ACU)

CLARIOstar®

Omega Series

Measuring mitochondrial function and glycolytic flux in 3D cell cultures

Conn Carey and James Hynes
Luxcel Biosciences

- **3D RAFT™ cell cultures were used in 96 well microplate format**
- **MitoXpress Xtra® – Oxygen Consumption Assay (HS Method) and pH-Xtra™ Glycolysis Assay were used to determine mitochondrial function and glycolytic flux**

Introduction

While, historically, 2D cultures have been the mainstay of *in vitro* assays, there is a developing interest in transferring to 3D models in an effort to increase the biological relevance of the measurement. By more closely reflecting conditions within the tissue, the hope is that such 3D models will help bridge the gap between *in vitro* and *in vivo* measurements thereby increasing the usefulness of the in vitro assay. Of equal importance to the aim of increasing biological relevance is the parameter measured. One of the most informative is to measure cell metabolism, whereby perturbed metabolism or mitochondrial function is probed without disrupting the 3D structure. This can be achieved in microtitre plate format using the *MitoXpress®-Xtra HS* and *pH-Xtra™* products from Luxcel Biosciences.

MitoXpress®-Xtra HS measures oxygen consumption and therefore informs specifically on mitochondrial function, while pH-Xtra™ measures extracellular acidification and is therefore a convenient measure of glycolytic flux. Here we demonstrate the application of these probe technologies to 3D cultures generated using the *RAFT™* system from TAP Biosystems. *RAFT™* facilitates the convenient production of consistent collagenbased structures. This, in conjunction with *MitoXpress®-Xtra HS* and *pH-Xtra™* facilitates detailed microplate-based measurements of metabolic activity of 3D cultures without disrupting the integrity of the 3D structure. Measurements are conducted on the FLUOstar Omega microplate reader from BMG LABTECH.

Materials & Methods

- 3D RAFT™ cultures including black walled clear bottom 96-well cell culture microplates, (TAP Biosystems)
- MitoXpress® Xtra-Oxygen Consumption Assay (HS Method), (Luxcel Biosciences)
- pH-Xtra™ Glycolysis Assay, (Luxcel Biosciences)
- FLUOstar® Omega microplate reader, (BMG LABTECH)
- DMEM and culture media were obtained through usual distribution channels

Plate Preparation

3D RAFT cultures were prepared with either A549 or HepG2 cells (data not shown) at the indicated density in 240 µl DMEM/Collagen solution on a 96-well plate. RAFT cultures were formed as per manufacturer's protocol (Fig. 1).

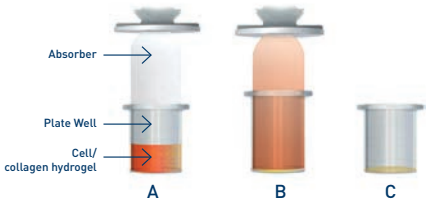

Fig. 1: RAFT Process Principle.

(Fig. 1A) Cells and neutralized collagen were mixed and pipetted into one well. After 15 min incubation at 37°C a hydrogel is formed. Medium was absorbed from the hydrogel (Fig. 1B) to increase concentration of collagen and cells to *in vivo* conditions. The process is completed (Fig. 1C) in less than 1 hour and results in a structure that is about 120 µm thick.

Oxygen Consumption Measurements

Culture medium was removed after a desired culture period and 150 µl of MitoXpress®-Xtra stock solution prepared in prewarmed DMEM was added. 1 µl of compound (150x) was added to appropriate wells. All wells were then sealed by adding 100 µl prewarmed HS mineral oil to prevent the back diffusion of ambient oxygen. The plate was then measured kinetically on a FLUOstar Omega for 90-120 minutes at 37°C.

Extracellular Acidification Measurements

Three hours prior to measurement the RAFT cell culture plate was placed in a CO_2 free incubator at 37°C, 95% humidity, in order to remove CO_2 from the plate material. Media was removed and 2 wash steps were performed using the Respiration Buffer (0.5 mM KH_2PO_4, 0.5 mM K_2HPO_4, 20 mM Glucose, 4.5 g/L NaCl, 4.0 g/L KCl, 0.097 g/L $MgSO_4$, 0.265 g/L $CaCl_2$). Finally 150 µl of Respiration Buffer containing pH-Xtra™ probe at the recommended concentration was added to each well. The plate was then measured kinetically.

FLUOstar Omega/CLARIOstar® instrument settings

Measurement method:	Time-resolved fluorescence (Omega: TR-F Optical attachment installed)
Measurement mode:	Plate Mode Kinetic
Measurement time:	120 min (data points every 2 min)
Measurement temperature:	37°C

Dual chromatic using the following windows:

Rev. 09/2014
Keywords: **2D cells, 3D cells, Glycolytic flux, Luxcel, MitoXpress, Oxygen consumption, pH**

	Excitation/ Emission filters	Integration start/time (µs)
MitoXpress®-Xtra	1 TREx L + 655-50 (Omega) + 645-20 (CLARIOstar)	30/30
	2 TREx L + 655-50 (Omega) + 645-20 (CLARIOstar)	70/30
pH-Xtra™ Glycolysis Assay	1 TREx L and 615-BP10	100/40
	2 TREx L and 615-BP10	300/40

Results & Discussion

Sample oxygen consumption profiles are presented in Figure 2.

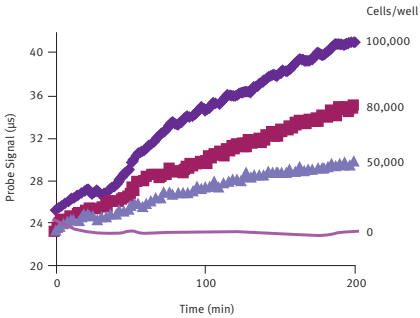

Fig. 2: Oxygen consumption profiles from A549 RAFTTM cultures of increasing cell density.

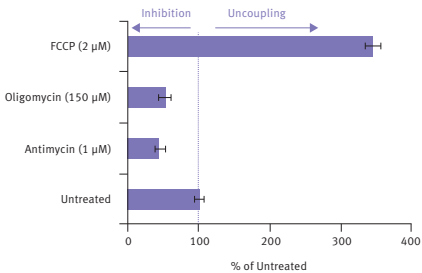

Fig. 3: Relative effect of drug treatment on cultures plated at 80,000 cells/well.

Increasing the density of cells in the 3D matrix results in increased rates of oxygen consumption, with strong signal changes observed across the cell concentrations tested. Fig 3 shows the effect of treatment on cells within the 3D structure where ETC activity has been pharmacologically modulated. Sample acidification profiles are presented in Figure 4 with significant acidification observed for untreated cells.

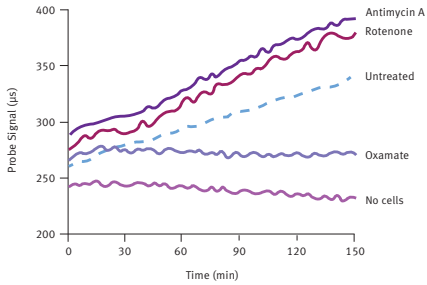

Fig. 4: Extracellular acidification profile from A549 RAFT cultures, using treated and untreated cells.

Acidification is inhibited almost completely on treatment with oxamate indicating that the acidification derives from the production of lactic acid. Treatment with antimycin however causes a significant increase in acidifications the cell increases glycolytic flux in order to maintain cellular ATP supply.

Conclusion

MitoXpress Xtra® – Oxygen Consumption Assay and pH-Xtra™ Glycolysis Assay, provide a convenient, sensitive and high throughput measure of mitochondrial function, metabolism and cellular energy flux, when combined with the FLUOstar Omega multimode plate reader. These data illustrate the applicability of these assays to the study of cellular function in a complex multicellular 3D RAFT™ culture system without disrupting the integrity of the 3D structure. The FLUOstar Omega is enabled with menu selection for easy instrument set-up and data analysis, including the ability to input calibrations to generate O_2 and H^+ scales.

Assessing epigenetic enzyme activity using HTRF® epigenetic assays from Cisbio with the PHERAstar® *FS* from BMG LABTECH

Thomas Roux[1], Rebecca LaRose[1] and Carl Peters[2]
[1] Cisbio [2] BMG LABTECH

- **HTRF assay to detect histone methyltransferase G9a activity can be used to screen for inhibitors in 384-well format**
- **PHERAstar® *FS* one flash fly mode improves read times which will increase throughput**

Introduction

Epigenetics is an emerging arena of science in which changes in the regulation of gene activity and expression are not dependent on the sequence of genes. Instead the changes in expression are the result of DNA methylation or modification of histones. These modifications are essential to the normal developmental process and the results can be devastating when the epigenetic process is disrupted leading to cancer and autoimmune disease. The realization of the importance of epigenetic regulation has led to the search for drugs which are capable of targeting the enzymes that add or remove epigenetic modifications. In order to perform screening to identify compounds which affect epigenetic modifying enzymes it is necessary to establish a reliable assay which is capable of monitoring the activity of these enzymes. Cisbio has made available a number of assays that have been verified to detect the activity of the enzymes involved in histone modifications. These enzymes fall into two broad classes:

Writers: enzymes that add methyl or acetyl groups
Erasers: enzymes that remove methyl or acetyl groups

Here we describe the verification of the performance of a Cisbio HTRF® based assay using the PHERAstar *FS* from BMG LABTECH.

Assay Principle

The assay is a Histone H3K9 monomethylation assay that uses a biotinylated peptide that comprises residues 1-21 of histone H3 in which lysine 9 is un-methylated as a substrate (Figure 1).

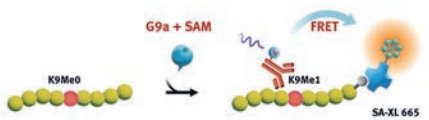

Fig. 1: HTRF® G9a Histone H3K9 mono-Methylation Assay Principle.

The assay is performed without washing in a single well using an enzymatic step followed by a detection step. In the enzymatic step substrate, enzyme G9a, and S-(5'-Adenosyl) L-methionine chloride (SAM) are combined. During incubation, active enzyme will transfer a methyl group to the lysine residue resulting in methylated substrate. The amount of modified substrate is detected via addition of a Eu^{3+}-cryptate labeled anti- H3K9 me1 detection antibody and XL665-conjugated streptavidin. The antibody will bind specifically to the methylated substrate and fluorescence resonance energy transfer will be observed between HTRF donor (antibody) and acceptor (XL665).

Materials & Methods

- H3K9 mono-methylation detection reagents and protocol (Cisbio)
- 384 well, small volume, white microplates (Greiner)
- G9a (Reaction Biology Corp.)
- H3K9 peptide substrate (AnaSpec)
- SAM & SAH (Sigma)
- UNC0646 & BIX01294 (R&D Systems)
- PHERAstar *FS* (BMG LABTECH)

To perform the enzymatic step working solutions were prepared in enzymatic buffer (50 mM Tris-HCl, pH 8.8, 10 mM NaCl, 4 mM DTT, 0.01% Tween 20). The reaction was assembled in a 384 well small volume plate in the following order:

- 4 µl inhibitor or enzymatic buffer
- 2 µl G9a (incubate for 5 minutes at room temperature)
- 4 µl substrate/SAM mix (seal plate and incubate at room temperature)
- 10 µl of detection mixture

After addition of the detection mixture the plate is sealed and incubated at room temperature for at least 1 hour. After 1 hour signal is stable for an extended period. Detection was performed with the PHERAstar *FS* using standard HTRF protocol settings.

Data analysis
The HTRF ratio (Signal at 665nm / Signal at 620nm; the result is multiplied with 10,000) is automatically calculated by the MARS Data Analysis Software.

Results & Discussion

Previous results indicated optimal performance by using a G9a concentration from 0.125-0.25 nM in 40 to 60 minute reaction time. Therefore the experiments described here employ a G9a concentration of 0.12 nM and a 40 minute reaction time. To verify performance a substrate titration was performed to determine the Km for the peptide in this experimental protocol. The results of this titration experiment are shown in Figure 2.

Rev. 05/2014
Keywords: **Enzyme inhibition curve, Epigenetics, Histone methyltransferase, HTRF, Laser (TRF)**

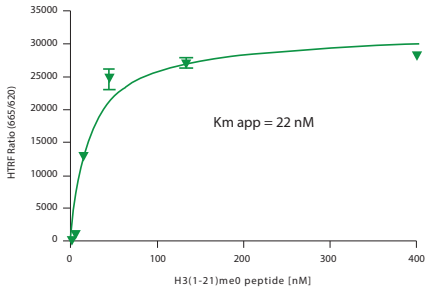

Fig. 2: **Peptide Substrate Titration** The apparent Km value for the peptide substrate was determined with the enzyme concentrations and reaction time described above and concentration of 15 mM SAM was used. Serial dilutions of the biotinylated H3K9 (1-21) me0 substrate were prepared which span from 400 to 2 nM. The streptavidin XL-655 concentration in the detection reagent was varied based on the peptide concentration to keep a constant ratio of 1:4 (streptavidin XL-655: peptide). An apparent Km value of 22 nM was determined from this experiment using a Michaelis Menten plot.

Final validation of the G9a H3K9 methylation assay was obtained by measuring the activity of known G9a inhibitors (Figure 3).

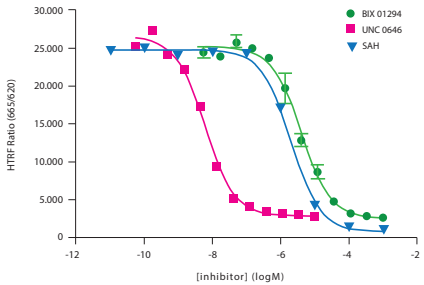

Fig. 3: Enzyme inhibition curves for three G9a inhibitor compounds Inhibitors were serially diluted and pre-incubated with G9a for 5 minutes. The enzymatic reaction was initiated with the addition of 15 µM SAM and 40 nM biotinylated H3 (1-21) me0 peptide.

IC_{50} values were calculated from enzyme inhibition curves similar to those shown in Figure 3. The IC_{50} results, are presented in Table 1. Table 2 compares the read times of the PHERAstar *FS* when flash number is varied.

Table 1: IC_{50} values for three different G9a inhibitors.

Inhibitor	IC_{50}
BIX 01294	4.1 µM
UNC 0646	6.2 nM
SAH	1.9 µM

Table 2: Read times for varying TRF laser conditions in a 384 well plate.

Laser mode:	Precise (27 flashes)	Rapid (7 flashes)	Flying (1 flash)
Read time:	2m32sec	48 sec	21 sec

Conclusion

- The Cisbio HTRF® G9a Histone H3K9 mono-Methylation Assay provides an excellent platform to screen for inhibitors of these enzymes with a central role in epigenetic regulation
- Assay performance on the PHERAstar *FS* is verified using substrate, cofactor and inhibitor titrations
- One flash fly mode significantly improves read times which will increase throughput
- Excellent performance in one flash fly mode can be attributed to:
  - UV laser as an excellent excitation source
  - HTRF® specific modules which provide true simultaneous dual emission detection
  - Matched PMT's designated for TRF detection

PHERAstar® *FSX*
PHERAstar® *FS*

CLARIOstar®

Omega Series

LanthaScreen® TR-FRET tyrosine kinase and protein kinase C assay

Randy Hoffman[1], Megan Buros[1], Kevin Kupcho[1] and E.J. Dell[2]
[1]Invitrogen Corporation [2]BMG LABTECH

- **Dual wavelength detection of terbium and fluorescein using dedicated optic settings**
- **Strong and long-lasting donor signal minimizes background**
- **Rapid and reliable detection of PKC´s and tyrosine kinase activity**

Introduction

Protein Kinase C (PKC) enzymes are a diverse family of enzymes that under specific signaling conditions phosphorylate proteins on serine and threonine amino acids. PKCs are involved in many cellular functions including neuronal activity, cell growth, cell proliferation, and cell movement, as well as in several diseases including cardiovascular, cancer, diabetes, and Alzheimer's.Therefore a TR-FRET screening assay, such as the one described here, can aid in further elucidating PKC's function.

Tyrosine kinases (TKs) are a diverse family of enzymes that under specific signaling conditions phosphorylate proteins on the amino acid tyrosine. TKs are involved in many cellular functions such as cell division and cell proliferation, as well as in several diseases including cancer and diabetes. Tyrosine kinases play important roles in cell growth, thereby making them important drug targets in cancer therapy. For that reason a TR-FRET screening assay, such as the one described here, can aid in elucidating specific inhibitors for tyrosine kinases.

Assay Principle

A fluorescein labeled poly-GT or poly-GAT (Glu, Ala, Tyr) substrate is incubated with a tyrosine kinase and ATP. For protein kinase C assays a fluorescein labeled PKC substrate is incubated with PKC and ATP. Subsequently, a terbium labeled antibody that binds to the phosphorylated form of the substrate is added. When the antibody interacts with the phosphorylated substrate, FRET will occur between the terbium label (emits at 490 nm) and the fluorescein moiety (emits at 520 nm) (Figure 1).

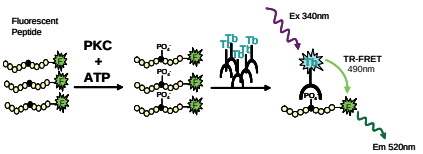

Fig. 1: LanthaScreen™ TR-FRET assay principle for a tyrosine kinase (TK) assay.

Measurements are taken at 490 and 520 nm and the 520/490 ratio is plotted versus the concentration of enzyme to determine an EC50 for the enzyme and its substrate.

Materials & Methods

- Invitrogen's Fluorescein PKC Substrate and PKC Kinase Buffer (5x), PKCα, Kinase Quench Buffer
- Invitrogen's LanthaScreen™ Tb-PKC Substrate Antibody
- Invitrogen's Fluorescein-poly-GT and Fluorescein-poly-GAT, Tyrosine Kinase Buffer, ZAP70, Screen Dilution Buffer
- Invitrogen's LanthaScreen® Tb-PY20 Antibody
- black Corning® low volume 384-well microplate

PKC Titration

Multiple PKC isoforms were titrated to determine optimal kinase concentrations for screening. The following protocol is an example of the conditions used to determine the EC50 of PKCα and its substrate.

A dilution series of PKCα, starting at a final concentration of 2.0 µg/ml, was incubated in the presence of 250 nM fluorescein-labeled PKC substrate and 20 µM ATP in a total volume of 10 µl in a microplate. After a 90-minute incubation at room temperature, 10 µl of TR-FRET dilution buffer containing 2X EDTA (20 mM) and 2X Tb-PKC antibody (1.0 nM) was added and mixed to create a final volume of 20 µl per well, a final 1X EDTA concentration of 10 mM, and a final 1X antibody concentration of 0.5 nM. After incubating for 60 minutes at room temperature, the plate was read on the BMG LABTECH microplate reader. Each data point represents the average of three wells.

Tyrosine Kinase Titration

To demonstrate the functionality of a universal tyrosine kinase assay using LanthaScreen™, both fluorescein-poly-GAT (Glu, Ala, Tyr) and fluorescein-poly-GT (Glu, Tyr) substrates were used with terbium labeled PY20 antibody.

A dilution series of kinase was incubated with 400 nM fluorescein labeled substrate and 200 µM ATP in a total volume of 10 µl in a microplate. After 60 mins of incubation at room temperature, 5 µl of TR-FRET dilution buffer containing 4X EDTA (60 mM) was added. Next, 5 µl of TR-FRET dilution buffer with 4X antibody (8 nM) was added and mixed to create a final volume of 20 µl per well, a final 1X EDTA concentration of 15 mM, and a final 1X antibody concentration of 2 nM. After 60 mins of incubation at room temperature, the plate was read in the microplate reader.

Rev. 12/2014
Keywords: **Fluorescein, LanthaScreen, PKC, Terbium, Tyrosine kinase**

Instrument settings

	FLUOstar®/ POLARstar® Omega	CLARIOstar®	PHERAstar *FS*
Detection mode	Time-resolved Fluorescence		
Method	Endpoint, Top optic		
Optic settings	Advanced TRF Optic Head Ex-Filter: TREx Em-FilterA: Em520 Em-FilterB: 490-10	Ex-Filter: Ex TR Em-FilterA: 520-10 Em-FilterB: 490-10	Lantha-Screen Optic module: 337 520 490
Integration start	100 µs		
Integration time	200 µs		

Results & Discussion

Figure 2 shows representative kinase titration curves for PKCα and PKCζ.

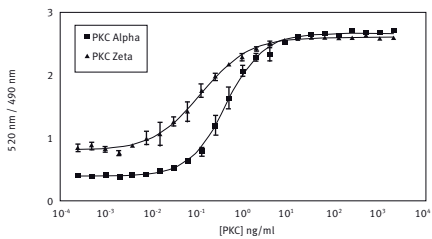

Fig. 2: LanthaScreen™ monitored on the PHERAstar *FS*: PKCα and PKCζ titrations

Table 1 shows statistical data for all kinases tested.

Table 1: LanthaScreen™ on the PHERAstar: statistics for PKC isoform titrations.

	PKCα	PK-CβI	PKC-βII	PKCδ	PKCε	PKCΘ	PKCζ
EC_{50} (ng/ml)	0.43	0.27	0.22	0.07	0.03	0.16	0.12
Min. Value	0.44	0.43	0.79	0.55	0.73	1.02	0.84
Max. Value	2.69	2.66	2.61	2.64	2.63	2.63	2.63
Max-min	2.25	2.23	1.82	2.09	1.90	1.61	1.79
Fold Diff (max/min)	6.1	6.2	3.3	4.8	3.6	2.6	3.1
Hill Slope	1.1	0.89	1.01	0.70	0.93	0.96	0.83
R^2	0.991	0.996	0.988	0.979	0.988	0.976	0.982
S:N	60	47	18	31	25	14	18
Z'-factor value	0.94	0.91	0.78	0.87	0.85	0.74	0.79

All PKC isoenzymes were titrated in a similar manner as PKCα. Regardless of "fold difference" or signal-to-noise ratios, all assays provided Z' values far greater than 0.5. This is due to the use of FRET-based, ratiometric data analysis, which leads to low standard deviations of the replicates. Both hill slope and R^2 measurements fall within acceptable limits. R^2 values approaching 1.0 indicate a perfect curve fit.
In figure 3 kinase titration curves for ZAP-70 are presented (Fig. 3A+B).

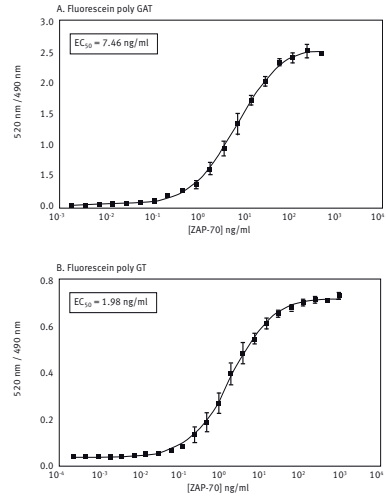

Fig. 3: LanthaScreen™ assay was measured on the PHERAstar *FS* for tyrosine kinase ZAP-70 at different concentrations and for different substrates. **A** The fluorescein-labeled poly GAT substrate is used. **B** The substrate is a fluorescein-labeled poly GT substrate.

The results show that use of a fluorescein-labeled substrate common for many TKs (poly-GT or poly-GAT) allows for the development of a "universal" TK assay using the LanthaScreen™ format.

Conclusion

Invitrogen's terbium-based LanthaScreen™ can easily be measured on BMG LABTECH instrumentation. The assay shows several advantages:

- The ratiometric nature of LanthaScreen™ assay eliminates well to well variation.
- The time-resolved nature allows for the use of fluorescein without the associated drawbacks of compound interference.
- The ability to use fluorescein simplifies assay development and costs.

HTRF® IP-One assay used for functional screenings

Hayley Jones[1] and Franka Ganske[2]
[1]MRC Technology London [2]BMG LABTECH, Germany

- **Single cell clones functional screening using fresh and frozen cells**
- **Functional HTS for the MRCT 100K compound collection**
- **Detection for screening is simplified using the HTRF optic module**

Introduction

G protein coupled receptors (GPCR) are a most prominent group of therapeutic targets. In most cases, the activation of GPCRs results either in the alteration of cellular cAMP level or in the release of calcium ions from intracellular stores. Various assays are available to determine receptor activation by direct or indirect quantification of these second messengers.
Cisbio Bioassays developed highly accurate HTRF® assays for measuring second messengers like IP1 or cAMP in HTS formats. This application note demonstrates the use of the IP-One HTRF® assay for a functional selection of clones from stably transfected recombinant CHO-M1 cells. Next to this, results from a high-throughput screen of Galanin receptor GALR2 are presented. This screen employed the MRCT 100K compound collection, a selection of drug-like molecules from commercial libraries which includes over 10,000 compounds which target protein-protein interaction.

Assay Principle

A robust HTRF® functional IP1 assay (Cisbio) was used for screening. The assay is based on a competitive immunoassay principle whereby free IP1 competes against IP1-d2 (HTRF® acceptor) for binding to anti-IP1 Cryptate conjugate (HTRF® donor). The signal is inversely proportional to IP1 levels in the cell with maximum FRET obtained in the absence of IP1 (Figure 1).

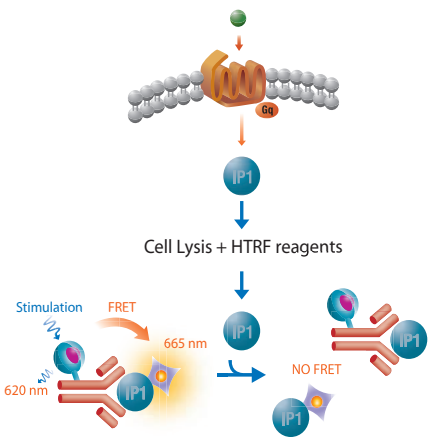

Fig. 1: Cisbio IP-One HTRF assay.

Materials & Methods

- CHO cells stably expressing GalR2 (GE Healthcare)
- Fresh and Frozen CHO-M1 Cell lines, CCS Culture Service, Germany
- IP-One HTRF® assay kit (Cisbio)
- White 384-well small volume plates (Greiner)

Single cell clone screening

CHO-K1 cells were transfected with an expression vector encoding the Gaq-coupled human muscarinic acetylcholine receptor M1 (CHRM1). Stably transfected monoclonal cell lines were isolated by selection with G418 in 384-well plates. In order to screen the clones for a functional agonist response, 10,000 cells were seeded into white 384-well plates and were left to adhere overnight. The next day the medium was discarded and cells from each clone were stimulated with agonist for 1 hour while identical cells in a control well were left untreated. Cells were prepared according to a standard freezing protocol and used in the assay as follows: After thawing, the cells were washed once in culture medium and resuspended in IP1 stimulation buffer. To compensate for the preincubation time of the fresh cells, which grew over night, the frozen cells were seeded at double density of 20,000 cells per well. The agonist was added to the cells in suspension immediately after seeding.

GalR2 screening

The assay was configured using CHO cells stably expressing GalR2. Pre-incubation of cells with a sub-maximal concentration of the galanin agonist sensitised the HTS to the simultaneous detection of both agonists and PAMs. The MRCT 100K compound collection was screened at a final assay concentration of 10 µM. 5 µl of cells/well (15,000 cells) were dispensed and 2.5 µl compound or buffer control was added to 384 well low volume white plates. Following a 30 min incubation at 37°C, 2.5 ml of galanin was added at a maximal concentration of 1 µM (EC100) or an above-minimal concentration of 3.16 nM (EC20). Test samples received buffer containing 0.1% BSA. Plates were incubated for 1 hr at 37°C.

HTRF® IP-One assay on PHERAstar® *FS*

The IP-One HTRF® assay was performed as recommended by Cisbio. Briefly, donor and acceptor were added consecutively to the cells followed by 1 hour incubation at room temperature. Plates were then put in the PHERAstar *FS* microplate reader. The 665 and 620 nm signals were measured simultaneously by using the HTRF specific optic module.

Rev. 12/2014
Keywords: **GPCR, HTRF, IP-One, Receptor, Screening**

Data calculation

Data normalization was performed by calculating the ratio of the raw data obtained at 620 and 665 nm:
Ratio = (665/620)*10000

Results & Discussion

Single cell clone screening

Upon stimulation with the physiological ligand acetylcholine (1 µM), recombinant CHO-M1 clones were functionally screened by the IP-One HTRF® assay. (Figure 2).

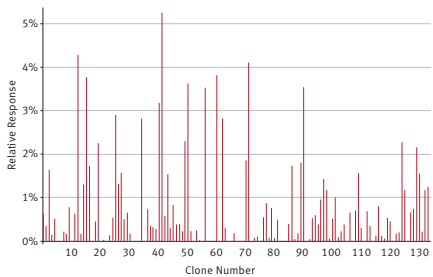

Fig. 2: Screening of single cell clones. Data are displayed in relation to the IPOne standard curve (29 µM IPOne = 100 %).

Out of 134 clones 27 were exhibiting a significant response to the agonist with different sensitivity. One selected cell line (clone B2) was taken to do a dose response curve with carbachol, a partial agonist (Figure 3).

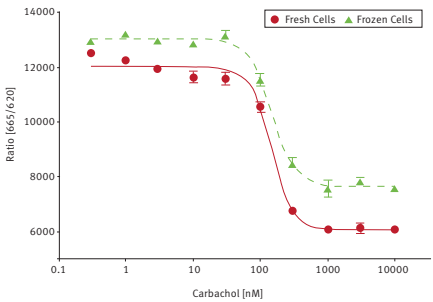

Fig. 3: Dose response of carbachol with cells from a growing culture (fresh cells) and from frozen cells.

Although the signal to background ratio was slightly reduced in frozen cells, similar EC50 values were determined (147 nM for fresh cells and 149 nM for frozen cells). The Z'factor of the assay with frozen cells was still very good (> 0.7).

GalR2 screening

The 665/620 ratio was expressed relative to EC100 and EC20 such that EC20 = 0% and EC100 = 100% (Figure 4). Hits were selected using a 30% response cut off or 30% above EC20.

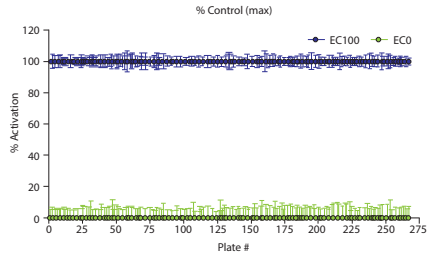

Fig. 4: Data were normalized to high (EC100) and low (EC20) controls.

The HTS assay performed well as indicated by the robust and consistent Z' data. For 85320 compounds screened, a mean Z' of 0.72 (± 0.05) was obtained. The number of hits is presented in Table 1.

Cutoff (%)	# Hits
30	250
40	117
50	65
60	29
70	13
80	9
90	4
100	1

The screen gave a fairly low hit rate (0.3 % at 30 % activity).

Conclusion

The IP-One HTRF® assay from Cisbio proved to be a fast and reliable method to directly screen a large number of samples using the PHERAstar *FS* microplate reader. A robust assay could be established using fresh cells from a growing culture as well as Frozen Instant Cells.

Miniaturization of an HTRF methyltransferase assay that detects histone modifying activity

Bonnie Edwards[1] and Carl Peters[2]
[1]Labcyte, Sunnyvale, CA [2]BMG LABTECH, Cary, NC

- **Assay miniaturization enables high throughput screening for compounds that impact epigenetics**
- **Excellent data quality (Z´> 0.88) was achieved in just 2 µl assay volume**

Introduction

Histone modifying enzymes are important epigenetic targets in drug discovery and cancer research. Some of the enzymes of interest are methyltransferases. Their activity leads to methylated histones, which affect the regulation of gene transcription. The methyl group is often provided by SAM (S-adenosyl-L-methionine). SET7/9 is a methyltransferase that uses SAM as a cofactor in order to methylate histone H3. In this way SET7/9 is able to modulate p53 activity in a human cancer cell line, indicating the importance of that enzyme in the process of human tumor formation.
In this application note we show the use of the EPIgeneous Methyltransferase Assay kit from Cisbio. The homogeneous approach of the HTRF technique enables the use of the kit for high throughput screening purposes. To limit the associated costs miniaturizing to significantly lower assay volumes was done. This requires very precise instrumentation and was performed with the help of the Echo liquid handler from Labcyte that uses acoustic dispensing (down to 2.5 nl) to accurately transfer kit reagents, enzyme, substrate, and compounds.

Assay Principle

The assay consists of two steps. In the enzymatic step the substrate is incubated with the enzyme in presence of compounds. Cofactor SAM is added to start the reaction. The result will be a methylated substrate while SAM is converted into SAH (S-adenosyl-L-homocysteine). In the second step detection reagents are added that contain an antibody specific to SAH. The antibody is labeled with Lumi4-Tb cryptate. To this d2-coupled SAH is added that competes with the SAH formed during the reaction for the antibody binding sites (Fig. 1).

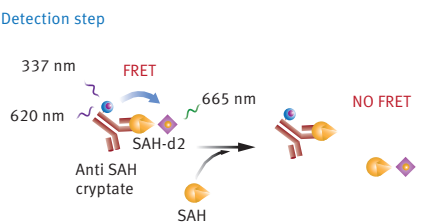

Fig. 1: Epigeneous Methyltransferase Assay Kit (Detection Step). If there is no SAH converted from SAM during the enzymatic reaction Anti-SAH will bind to SAH-d2 leading to a large HTRF signal. If SAH is present in the well after the enzymatic reaction is finished, SAH will compete with SAH-d2 on the binding sites of the antibody leading to a decrease in TR-FRET signal.

Materials & Methods

- EPIgeneous Methyltransferase Assay Kit from Cisbio
- 384-well white, low volume assay plates from Greiner
- 1536-well white assay plates from Corning
- SET7/9 from BPS Bioscience
- Biotinylated histone H3 (1-21) from AnaSpec
- (R)-PFI 2 hydrochloride from Tocris Biosciences
- SAM and SAH from Sigma-Aldrich
- Echo® liquid handler from Labcyte
- PHERAstar® *FS* microplate reader from BMG LABTECH

Reagent preparation
SAM was dissolved in 5 mM H_2SO_4/10% ethanol (v/v) in water, to make a 30 mM solution. Assay buffer consisted of 50 mM TRIS-HCL pH 8.8, 10 mM NaCl, 1 mM DTT, 0.01% Tween-20. HTRF detection buffer one and two were ready to use after thawing at room temperature.

Standard curves
For pretest purposes SAM/SAH standard curves were created. These standard curves mimic the methyl transfer in the enzymatic reaction. 16 standards were prepared in assay buffer, starting with 1 µM SAH (1:2 dilution). Triplicates of each dilution were transferred into 384-well or 1536-well microplates. Detection reagents were added and after 60 min of incubation the plates were read in the PHERAstar *FS*.

Enzyme titration
Half-log dilution SET7/9 titration curve was prepared in Echo-qualified, 384-well source plates. 400 nl of the different enzyme concentrations were transferred to 384-well assay plates in triplicates. 200 nl assay buffer, 200 nL 800 nM biotinylated histone and 200 nl 1 µM SAM were added, plates sealed and incubated at room temperature for 60 min. After incubation, detection buffer one was added to stop the enzymatic reaction. After further 10 minutes of incubation, anti-SAH lumi-Tb cryptate and SAH-d2 prepared in detection buffer two were added. After the final 60 min incubation step the microplate was read in the PHERAstar *FS*.

Inhibitor dose response curve
A 12-point half-log dose response series of Set7/9 inhibitor (R)-PFI 2 hydrochloride was prepared in DMSO, starting at the highest concentration of 100 µM. Inhibitor was transferred into 384-well and 1536-well plates followed by assay buffer, 40 nM enzyme, 800 nM biotinylated histone, and 1 µM SAM. All reaction and incubation steps were done as described for the enzyme titration. After final incubation step the microplates were measured in the PHERAstar *FS*.

Rev. 04/2015
Keywords: **Epigenetics, Histone, HTRF, HTS, Miniaturization**

All transfer and pipetting steps were done using the Echo liquid handler.

PHERAstar FS instrument settings

Detection mode:	TRF, endpoint
Optic Module:	HTRF 337 665 620
Integration start time:	60 µs
Integration time:	400 µs

Results & Discussion

In Fig. 2 SAM/SAH standard curves are shown that were measured either in 384-well or in 1536-well format. The results indicate that the assay is applicable in different plate formats. It is also shown that a final volume of as little as 1.25 µl can be used.

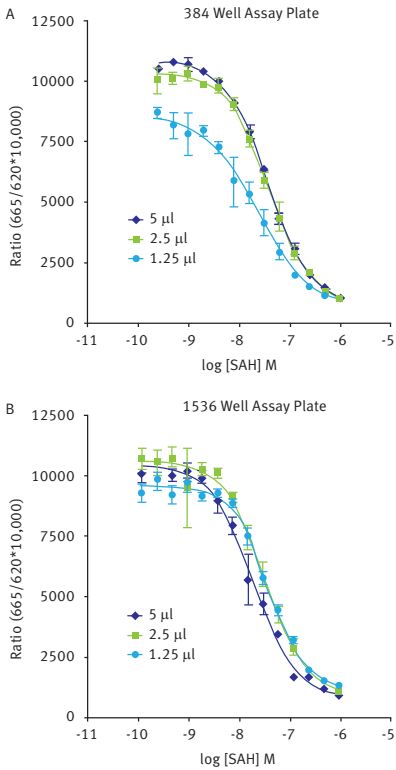

Fig. 2: SAM/SAH standard curves in 384-well **(A)** and 1536-well **(B)** assay plates using different volumes.

A SET7/9 enzyme titration was done in order to find optimal enzyme concentrations. Fig. 3 shows the titration result. From this an enzyme concentration of 40 nM was determined to be optimal to use for subsequent experiments.

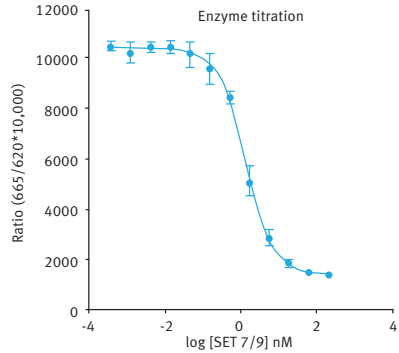

Fig. 3: SET7/9 enzyme titration in the enzymatic reaction volume of 1 µl.

For the SET7/9 inhibitor IC_{50} values could be calculated from the inhibitor dose response curve (Fig. 4).

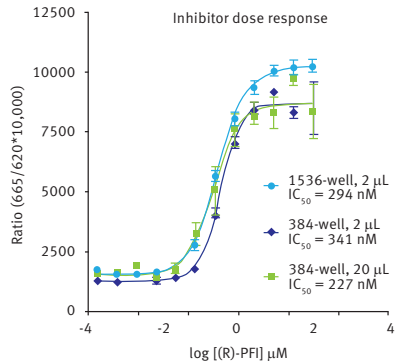

Fig. 4: (R)-PFI 2 hydrochloride inhibitor titration with SET7/9 enzyme.

Miniaturization of the assay was very successful indicated by excellent data quality. The Z´ factors for the miniaturized assays were > 0.88 and agreed very well to the assay parameters obtained for the standard 20 µl assay (Table 1).

Table 1: Comparison of Z´ factors and signal to background (SIB).

Assay format	S/B	Z´factor
384-well (20 µl)	6.4	0.83
384-well (2 µl)	7.9	0.88
1536-well (2 µl)	7.3	0.93

Conclusion

The SET7/9 methyltransferase assay could be miniaturized 10 fold leading to significant cost savings on reagents, enzymes, substrates, and compounds.

PHERAstar® *FSX*
PHERAstar® *FS*

Detection of human tau protein aggregation

Delphine Jaga[1] and Franka Maurer[2]
[1]Cisbio Bioassays, France [2]BMG LABTECH, Germany

- **Detection and quantification of tau aggregates in brain tissue**
- **Kinetic of tau aggregation and dissociation**
- **Small sample size < 10 µl**

Introduction

The tau protein stabilizes microtubule structures in the brain. These structures are supporting the nutrient transport between neurons. Abnormal tau protein leads to collapse of structure and transport – plaques will be developed. This happens in patients that undergo neurodegeneration, e.g. in Alzheimer´s. The level of a patient´s tau protein can therefore be an indicator of a neurodegeneration disease state. To that end Cisbio developed a tau aggregation kit that can be applied to cell cultures, brain tissue extracts, and recombinant proteins.

Assay Principle

Tau aggregates are measured using a sandwich immunoassay, applying an anti-tau monoclonal antibody labeled either with terbium-cryptate or d2, ensuring assay quality reproducibility and signal quality. The specific HTRF signal that is generated is proportional to the amount of tau aggregates (Fig. 1).

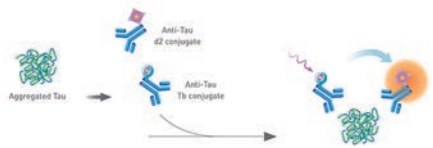

Fig. 1: HTRF® tau aggregation assay principle.

When terbium-cryptate and d2 are in close proximity, the excitation of the HTRF donor with a laser or a flash lamp will lead to an energy transfer (FRET) to the HTRF acceptor. The result is a specific FRET signal at 665 nm. At a wavelength of 620 nm the donor emission is measured.

Figure 2 shows the steps to carry out the tau aggregation assay protocol.

Fig. 2: HTRF® mix and measure protocol.

Material & Methods

- TAU aggregation assay kit (#6FTAUPEG) from Cisbio including a white 384-well low volume microplate
- PHERAstar® *FS* microplate reader from BMG LABTECH

Only 10 µl of sample is needed and given into a well of a 384-well plate. Next 5 µl anti-tau-d2 antibody and 5 µl anti-tau-tb antibody are added. After incubation of 2 hours at room temperature the HTRF signals are measured in a PHERAstar *FS* microplate reader.

Instrument settings

Detection Method:	Time-resolved fluorescence, endpoint
Optic:	Top optic
Optic Module:	HTRF 337 665 620
Integration start:	60 µs
Integration time:	400 µs
Excitation source:	Laser or flash lamp
Simultaneous dual emission:	Yes

The results of the two emission signals will be automatically converted into HTRF Ratio or DeltaF% values by the MARS Data Analysis Software.

Results & Discussion

Assay specificity and linearity

Figure 3 shows that the assay can clearly distinguish between samples containing tau chemically aggregated in comparison to non aggregated tau.

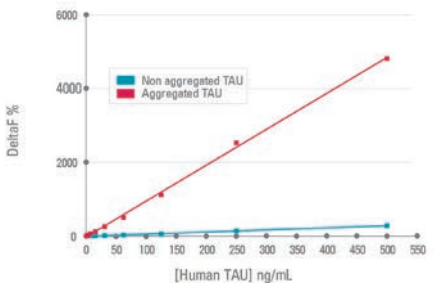

Fig. 3: DeltaF% values obtained for aggregated tau and non aggregated tau.

While non aggregated tau does not show a significant increase, HTRF values of aggregated tau samples increase with concentration. There is a linear relationship for tau aggregation.

Rev. 09/2015
Keywords: **Aggregation, Alzheimer's, HTRF, Neurodegeneration, Tau**

Kinetics of tau aggregation

Chemical aggregation was used to receive aggregates of recombinant full length human tau protein. To evaluate kinetic parameters, 5 samples were prepared in parallel and the reaction was stopped at different time points (between 1 and 24 hours) (Fig. 4).

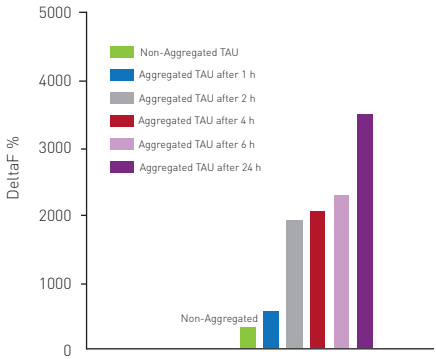

Fig. 4: Kinetics of tau aggregation.

This assay system can also be applied for monitoring a kinetic of dissociation after compound addition.

Tau aggregation on transgenic mouse brain extracts

Tau aggregation was determined in brain extract samples from transgenic mice (Tau/PSEN2/APP). From these mice it is known that they develop neurodegeneration pathology over time (Fig. 5).

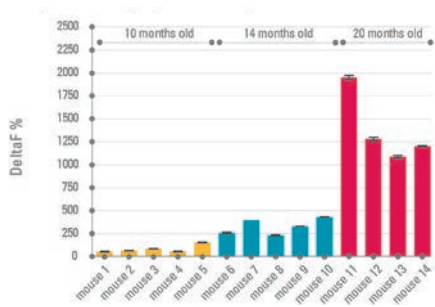

Fig. 5: Tau aggregation of transgenic mouse brain extracts.

The figure shows that not only tau aggregates in late stages can be detected with the assay (red bars). The assay is specific enough to discriminate between early stages of tau fibrillization (yellow and blue bars).

Conclusion

Tau aggregation was successfully measured on the PHERAstar *FS* microplate reader. While the sample volume is very small (< 10 µl) the assay is applicable for detection of early stage fibrillization. Using the HTRF technology offers the possibility to measure a sample several times without the effect of bleaching. This property of the HTRF chemicals allow for kinetic measurements to monitor tau aggregation in real time.

PHERAstar® *FSX*
PHERAstar® *FS*

Real-time measurement of intracellular O_2 in mammalian cells

Franka Maurer[1] and Conn Carey[2]
[1]BMG LABTECH, Germany [2]Luxcel Biosciences, Cork, Ireland

- **NEW O_2-sensitive cell-penetrating nanoparticle probe MitoXpress® Intra – Intracellular Oxygen Assay**
- **Cytosolic O_2 tension significantly influences signal transduction and cellular metabolism**
- **Dedicated measurement protocols, [O_2] scale conversion and data analysis with one mouse click**

Introduction & Assay Principle

As illustrated in recent publications, the level of available oxygen in the cell significantly influences cell physiology, signal transduction and cellular response to drug treatment.[1,2,3] In spite of this knowledge, the majority of *in vitro* studies culture and study cells at ambient oxygen – ignoring the oxygen gradient between the atmosphere and the medium and between the medium and the intracellular cell environment. To facilitate the quantification of cellular oxygenation Luxcel Biosciences has developed the MitoXpress Intra – Intracellular Oxygen Assay kit, based on a proprietary O_2-sensitive cell-penetrating nanoparticle probe.[4]

In this application note we show how a MitoXpress Intra assay is performed on the CLARIOstar microplate reader equipped with an atmospheric control unit (ACU). The ACU is a microprocessor-controlled unit that can regulate CO_2 and O_2 within the reader to reproduce the optimal physiological as well as hypoxic conditions needed for live cell-based assays. The MitoXpress Intra nanoparticle probe is taken up by cells during an overnight loading period and responds in real time to any changes in intracellular oxygen concentration in both 2D culture as well as a wide range of 3D systems, including Matrigel, RAFT™, microtissues, Alvetex®, Mimetix® and other scaffolds. Oxygen quenches the phosphorescent emission from the probe, such that phosphorescence is proportional to [O_2].

Cellular respiration can reduce the levels of intracellular oxygen concentration, creating a local oxygen gradient. In the specific example shown here (Fig. 1) for cells cultured under ambient oxygen, the intracellular [O_2] measured using MitoXpress Intra was found to be ~14% for metabolically inactive cells and ~7% for metabolically active cell types. However, when the environmental O_2 concentration is reduced to ~6% using an ACU, the intracellular [O_2] falls to ~4.5% for metabolically less active cells, and is close to anoxia for metabolically active cells.

As the real oxygen concentration experienced by cells in culture is a function of environmental O_2 concentration, cell metabolism and seeding density, MitoXpress Intra provides the ideal tool to intelligently modulate these parameters to achieve a desired and specific intracellular oxygen concentration. Similarly, MitoXpress Intra is an ideal tool to monitor real-time changes in intracellular [O_2] in response to treatments that perturb mitochondrial function and cell metabolism.

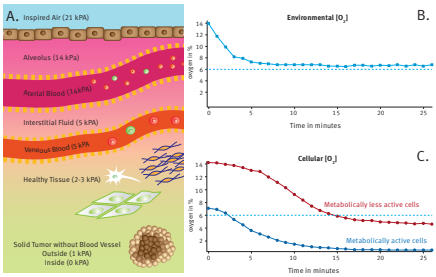

Fig. 1: (A) Schematic illustration showing changing O_2 tension in tissues. (B) *In vitro* cell culture environmental [O_2] set to ~6% using an ACU and (C) corresponding cellular [O_2] measured by MitoXpress Intra for metabolically active (blue) and metabolically less active cells (red).

Material & Methods

- MitoXpress Intra (MX-300, www.luxcel.com)
- CLARIOstar® microplate reader equipped with ACU

Instrument settings

Detection Mode:	Dual-read, TR-F, bottom reading
Method:	Plate mode kinetic
No. of cycles:	300
Cycle time:	60 seconds
No. of flashes per well:	100
Well multichromatics:	Yes
Injection volume:	Various using onboard injectors
Target temperature:	37°C
Target O_2 concentration:	Various
Target CO_2 concentration:	Set off

Optic Settings

	Excitation	Dichroic	Emission	Int. start	Int. time	Gain
1	F: Ex TR	F: LP TR	F: 645-20	30 µs	30 µs	1900
2	F: Ex TR	F: LP TR	F: 645-20	70 µs	30 µs	1900

Data Calculation

A data transform by the MARS software is required to convert signal to lifetime and from lifetime to %O_2. The CLARIOstar and FLUOstar Omega readers come with pre-installed measurement protocols and [O_2] scale that allow the user single mouse click data conversion.

Results & Discussion

Cellular response to decreased ambient oxygen

Sample data are presented in Fig. 2 illustrating the effect of cell respiration on the O_2 concentration experienced

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Keywords: **3D cells, ACU Atmospheric Control Unit, Mitochondria, MitoXpress, Oxygen**

by liver cells grown within a 3D collagen culture (RAFT™). The ACU was used to create a stepwise series of atmospheric environments between 19 % and close to 0 % oxygen.

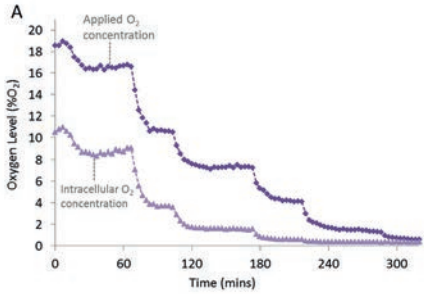

Fig. 2: Monitoring O_2 concentrations in samples containing 3D Hep2 cells in response to decreasing atmospheric O_2 conditions realized by the CLARIOstar ACU.

The initial oxygen concentration set by the microplate reader ACU is ~19 %, however the measured oxygen concentration of cells in the 3D culture is around 10 %. This reduced [O_2] is due to rapid local depletion of oxygen, consumed through cellular respiration by this metabolically active cell type. At each environmental oxygen concentration set, the actual [O_2] experienced by these liver cells is between 2 to 10 % lower. At the levels of environmental oxygen typically used by researchers studying hypoxia (~5%) it can be seen that the local [O_2] of the cells is close to zero.

Effect of compound addition

Sample data are presented in Fig. 3 and Fig. 4 illustrating the immediate and longer term effect from the addition of drugs that affect mitochondrial function and cell metabolism, in 2D and 3D culture, respectively.

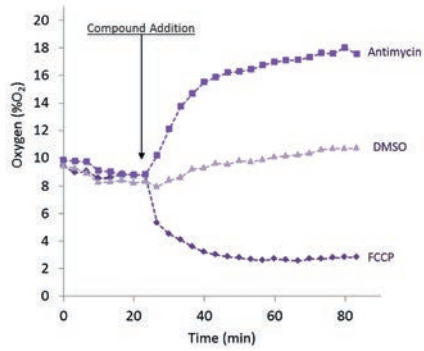

Fig. 3: Monitoring intracellular O_2 concentrations in a fully confluent monolayer of HepG2 cells.

Sequential compound additions are a feature of the CLARIOstar's onboard injectors, which offer the possibility to study opposing effects of different compounds on cellular [O_2] in the same well.

Cell respiration reduces the O_2 concentration from ambient to a baseline of ~10 % cells grown as a monolayer and to ~9% for cells grown in 3D culture. Increasing oxygen consumption rate by treatment with the mitochondrial uncoupler FCCP causes an acute and dramatic decrease in [O_2] to ~2-3 %, while complete inhibition of respiration by the mitochondrial inhibitor antimycin returns O_2 to ambient concentrations. Addition of DMSO serves as control and has no significant influence on oxygen concentration.

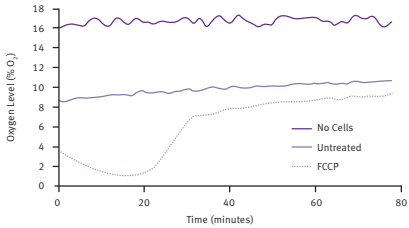

Fig. 4: Measuring the effect of drug treatment on cellular oxygenation, with HepG2 3D RAFT™ structures using MitoXpress Intra at 21% applied oxygen.

Conclusion

The physiological O_2 and substrate environment in which cells are cultured significantly affect signal transduction and cellular response to drug treatment. For the first time Luxcel's MitoXpress Intra – Intracellular Oxygen Assay kit provides researchers with an easy to use tool, with which they can measure the actual [O_2] of their cell culture system achieving greater understanding of the impact on cellular physiology.

The CLARIOstar equipped with an ACU is an excellent choice to measure intracellular [O_2]. The instrument is equipped with temperature and atmospheric control as well as shaking options for all requirements. These features are necessary for doing long term cell culturing while measuring alterations in the cells metabolisms caused e.g. by a decreased ambient oxygen content. Onboard injectors can be used to add compounds.

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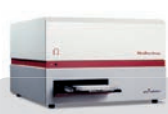
Atmospheric Control Unit (ACU)

CLARIOstar®



Omega Series

Development of a Rapid HTRF Insulin Assay

Z.J. Farino[1], T.J. Morgenstern[1], J. Vallaghe[2], N. Gregor[2], P. Donthamsetti[1], P.E. Harris[1], N. Pierre[2], R. Freyberg[3], F. Charrier-Savournin[2], J.A. Javitch[1] and Zachary Freyberg[1]
[1]Columbia University, NY [2]Research Dept., Cisbio Assays, France [3]Yeshiva University, NY

- **Direct measurement of insulin is necessary to further our understanding of insulin release**
- **The homogeneous HTRF Insulin assay proves to be suitable for HTS**

Introduction

When insulin secretion is not appropriately regulated it can lead to extensive metabolic problems which can result in diabetes mellitus[1]. Therefore, direct measurement of insulin is essential for the studies that will elucidate the mechanism of glucose stimulated release and the effects of dopaminergic modulators of insulin release[2].

Here we show the development of an insulin detection assay based on the homogeneous time resolved fluorescence (HTRF) detection method (Figure 1). Detection of this HTRF assay was assisted by the technology incorporated in to the PHERAstar *FS*, which is designed to optimize HTRF detection. This HTRF insulin assay provides quick and cost-effective results. The assay is further able to detect insulin from a variety of species. Performance was confirmed using insulin secreting cell lines and tissues, and tests indicate that this system will be suitable for detecting the effect of modulators on insulin secretion.

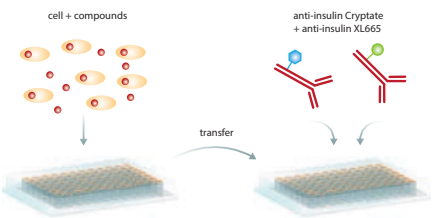


Fig. 1: Homogeneous HTRF Assay procedure. Secreted insulin in cells or supernatant is transferred to a new microplate, antibodies are added, incubated and read by the PHERAstar *FS* microplate reader.

Assay Principle

This HTRF insulin assay relies on two insulin binding antibodies (Figure 2). One antibody is labelled with a Europium cryptate donor and the second antibody is labelled with a near-infrared acceptor. When both antibodies are bound to insulin they are in close enough proximity for FRET (fluorescence resonance energy transfer) to occur. Because of the nature of the donor, a time delay before measurement start can be set that reduces transient interference from other assay components.

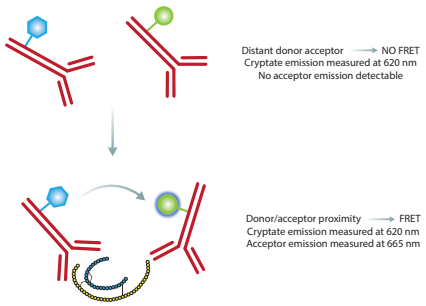


Fig. 2: Principle of the HTRF insulin assay. In the absence of insulin no FRET is occuring and only the donor will give a signal. The binding of both antibodies to insulin results in physical proximity, FRET occurs and acceptor emission can be detected. Ratiometric measurements reduce well to well variation.

Materials & Methods

- 96-well (half area) or 384-well, white plates (Greiner)
- Anti-insulin: Eu and anti-insulin: XL665 (Cisbio)
- Rodent insulin (ALPCO)
- Bromocriptine mesylate (Tocris)
- PHERAstar *FS* microplate reader

All other compounds were obtained from SigmaAldrich. For details on mouse pancreatic islet isolation, cell culture and the treatment of tissues and cells with insulin secretion modifying compounds please refer to Farino and Morgenstern *et al.*[2]

The HTRF insulin detection assay was performed by adding both anti-insulin antibodies to samples in a 1:1 ratio (total antibody volume: sample volume). After a 2 hour incubation at 25° C in pH 7 buffer the plate was read on a PHERAstar *FS*.

PHERAstar FS Instrument Settings:

Measurement type:	Time Resolved Fluorescence
Measurement mode:	Endpoint
Optic module:	HTRF (337/665/620)
Focal height:	adjust prior to test run
Integration start:	40 µs
Integration time:	100 µs
Excitation source:	flash lamp
Flashes/well:	100

Rev. 04/2016
Keywords: **HTRF, Insulin, Glucose, Dopamine, Receptor agonist**

Results & Discussion

The HTRF insulin assay was tested for the ability to detect insulin from a variety of species (Figure 3). The results indicate that the assay will be suitable for use with human cells and various animal models.

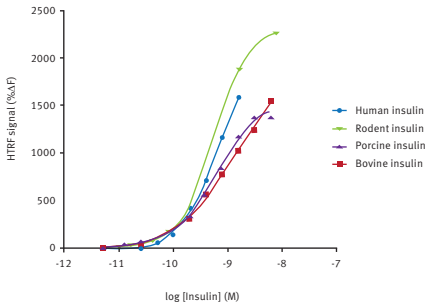


Fig. 3: Species cross-reactivity of antibodies. Data taken from Farino *et al.*[2]

No significant difference in HTRF signal were observed when insulin from humans, rodents, pigs or cows was tested across a range of concentrations from 0.01 – 10 nM (n = 3).

The suitability of this assay for high throughput screening was further assessed by the determination of the Z' – factor. Figure 4 shows the well-to-well variation of standards with different insulin concentrations.

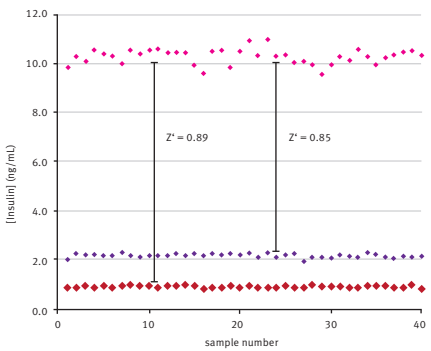


Fig. 4: Well-to-well variation of back calculated insulin concentrations. Data was first published in reference 3.

Results are based on 40 replicates and indicate that this assay is indeed suitable for HTS applications (Z´= 0.85, calculated between 2.5 ng/ml insulin and 10 ng/ml insulin samples).

To further illuminate the potential of this assay we tested the hypothesis that bromocriptine, a known dopamine D2/D3 receptor agonist might act directly on pancreatic islets. The results shown in Figure 5 indicate that bromocriptine can inhibit glucose stimulated insulin secretion in mouse pancreatic islets.

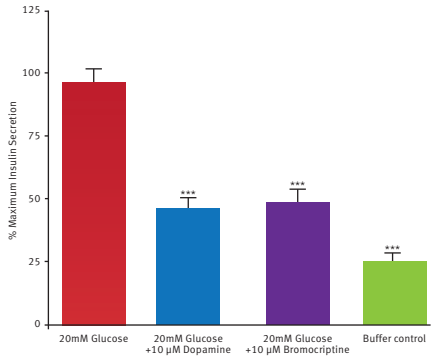


Fig. 5: Measurement of bromocriptine effect on insulin production in islets. Data are presented as % maximal insulin secretion in response to glucose. Bromocriptine inhibited insulin secretion by 67.4% ± 8.1% (n = 6). From Farino *et al.*[2]

Conclusion

The HTRF insulin assay is a homogenous assay that is straightforward and fast to perform. The results shown here indicate the suitability of this assay for medium/high throughput. Furthermore it can be performed at very low cost ($ 0.02 / sample)[2].

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CLARIOstar®

Fluorescence polarization discriminates green fluorescent protein from interfering autofluorescence

Andrew W. Knight
Gentronix Ltd., CTF Building, 46 Grafton Street, Manchester, M13 9NT

- **Novel use of fluorescence polarization (FP) optics**
- **Fluorescence polarization applied to yeast based genotoxicity assay**
- **Genotoxic species identified by expression of GFP, in spite of masking autofluorescence**

Introduction

Green fluorescent protein (GFP) has been widely adopted as a versatile marker for reporting on gene expression. However, fluorescence measurements of GFP (unless it is very highly expressed) are invariably contaminated with endogenous autofluorescence from the cells or media. This paper describes a novel use of the fluorescence polarization (FP) optics available on a BMG LABTECH microplate reader, which can dramatically increase the resolution of GFP fluorescence in the presence of unwanted autofluorescence. The reader has been used to distinguish the expression of GFP in yeast cells in an assay for genotoxicity, where the test compounds themselves were highly autofluorescent and would ordinarily mask GFP, making toxicity assessment impossible.

Theory

When GFP is illuminated with plane polarized light, because of its relatively large size and slow rotation, a high proportion of the emitted fluorescence remains polarized with respect to the excitation source. The degree of polarization (P), was found to be approximately 0.40 (400 mP) for GFP. Fluorescein, shows low fluorescence anisotropy due to its small size and fast rotation. Hence by taking the difference between the two polarized fluorescence measurements (Ipara - Iperp), a signal is obtained which is large for GFP, but small for naturally occurring autofluorescent species.

Experimental

Yeast cells are combined with serial dilutions of the compound to be tested in a microplate. The yeast (Saccharomyces cerevisiae) has been genetically modified to express GFP when the cell's DNA repair mechanisms are activated, upon exposure to a genotoxic compound.
Assessment of general cellular toxicity (cytotoxicity) is made simultaneously by measuring the degree of cell proliferation. A second yeast strain, not expressing GFP, is used as a control.
The BMG LABTECH microplate reader was used to make fluorescence measurements from the top of the plate (excitation filter = 485-12, emission filter = 520-30), and absorbance for cell density assessment (filter = 620 nm). The microplates used were Matrix Technologies, 96-well, black, clear bottomed plates. 75 µL of the serially diluted test compound was combined with 75 µL yeast cell reagent per well. The plate also contained various standard compounds and blanks. The microplates were and incubated at 25°C overnight. Since the toxicity of the test compound affects the final cell density achieved, the fluorescence reading is normalized for the number of cells present to form a "brightness reading". Thus:

$$\text{Brightness measurement by conventional method} = \frac{\text{Fluorescence}}{\text{Absorbance}}$$

$$\text{Brightness measurement by polarization method} = \frac{I_{para} - I_{perp}}{\text{Absorbance}}$$

Results & Discussion

The assay was performed by exposing 10 serial dilutions (from 32.5 µg/mL) of methyl methanesulphonate (MMS), a known genotoxic alkylating agent, to both the test and control yeast strains. The sample was spiked with 185 ng/mL fluorescein, an intensely fluorescent spectroscopic mimic of GFP, and a model autofluorescent compound in this study. GFP fluoresces at 517 nm and fluorescein at 512 nm.

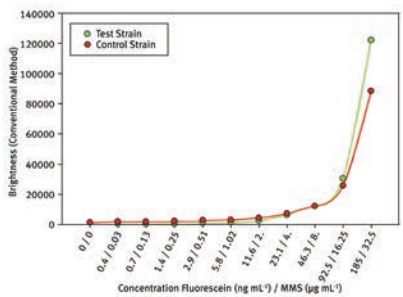

Fig. 1: Fluorescence of the control and test strains exposed to the genotoxin MMS spiked with a model interfering autofluorescent compound, fluorescein.

Using conventional fluorescence (Figure 1), both the test and control strains show increasing fluorescence with increasing compound concentration; meaning that the induction of GFP in the test strain is effectively masked, making genotoxicity assessment impossible.

Figure 2 shows that applying the fluorescence polarization method, the fluorescence signals from the test and control strains are now separated, as the fluorescence from the added fluorescein is removed. A dose dependant increase in GFP induction with the test strain is now evident with increasing MMS concentration.

Rev. 06/2007
Keywords: **Autofluorescence, GFP2, Green fluorescent protein, Genotoxicity, Proflavin**

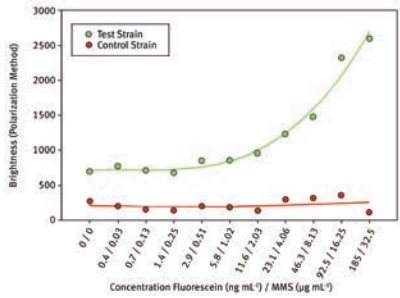

Fig. 2: Polarization of the control and test strains exposed to the genotoxin MMS spiked with a model interfering autofluorescent compound, fluorescein.

The method was used to analyze two compounds, that are highly fluorescent at the GFP wavelengths of interest. These were proflavin (513.5 nm) and methapyrilene (515.0 nm). Using conventional fluorescence, neither compound (Figures 3 and 4) can be reliably classified as a genotoxin.

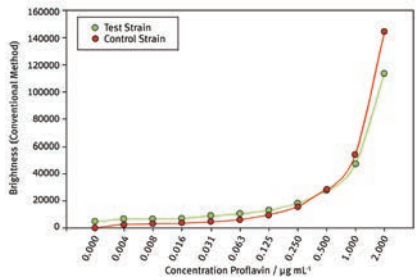

Fig. 3: Brightness of the control and test strains exposed to proflavin - measured using conventional fluorescence.

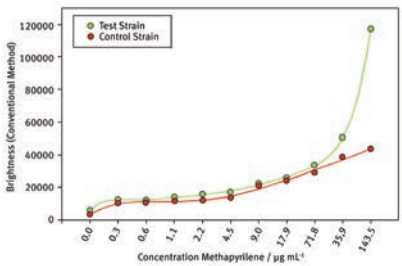

Fig. 4: Brightness of the control and test strains exposed to methapyrilene - measured using conventional fluorescence.

The fluorescence polarization discrimination method (Figures 5 and 6) clearly shows the induction of GFP in the test yeast strain compared to the non-expressing control strain; thereby confirming both compounds as genotoxic in this assay.

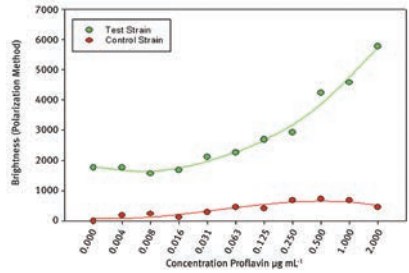

Fig. 5: Brightness of the control and test strains exposed to proflavin - measured using the new polarization method.

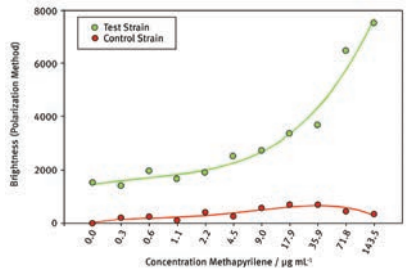

Fig. 6: Brightness of the control and test strains exposed to methapyrilene - measured using the new polarization method.

Conclusion

Exploiting the inherent fluorescence anisotropy of GFP, a novel method for discriminating GFP from interfering autofluorescent species has been achieved. The assay was performed using the polarization optics available on the BMG LABTECH microplate reader.
The method allowed both proflavin and methapyrilene to be identified as genotoxic species, which would not have been possible using conventional measurements.

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Identification of false positives in an fluorescence polarization screen

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- **Fluorescence Polarization screen configured on a BMG LABTECH microplate reader**
- **Rapid detection of false positives**
- **Identification of interfering compounds and non-specific inhibitors**

Introduction

Fluorescence polarization (FP) provides a useful tool with which to screen for inhibitors of the interaction of proteins with defined peptides.
FP gives a measure of the proportion of peptide found in the bound state in a homogeneous format. However compound interference and non-specific gross structural changes to the protein can give rise to a large number of false positives, which are only identified in later stage biochemical assays. Strategies to eliminate these at an early stage of screening will accelerate the hit to lead process.

Here we present data from an FP screen configured on a BMG LABTEC microplate reader for the interaction of the retinoblastoma tumor suppressor protein (pRB) with E2F peptide. Fluorescein-tagged E2F peptide was used to screen 10,000 small drug like molecules. Hit confirmation strategies based on fluorescence interference and specificity were developed.

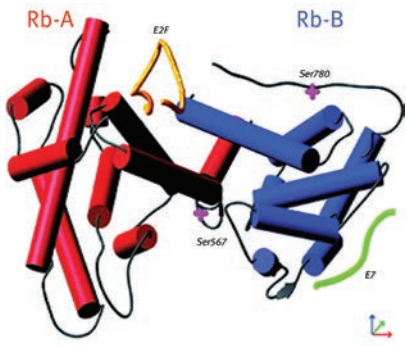

Fig. 1: Model of the interaction of pRb with E2F and E7 peptides.

An FP screen was configured for the interaction of recombinant pRb A/B domains with E2F peptide (depicted in yellow in figure 1). In addition, a second peptide binding site (E7, depicted in green), was utilised as an internal control for non-specific inhibitors. Fluorescein-E7 and rhodamine-E2F labelled peptides were used for hit confirmation.

Materials & Methods

Peptides were synthesised and fluoro-tagged. Before use peptides were dissolved in assay buffer (50 mM Tris HCl, pH 7.0, 100 mM NaCl, 10 mM DTT, 0.05% NP-40). Typical titration binding curves of pRb with the fluoro-labelled peptides are shown (mean±sem, n=3) in figure 2. Fluorescein-E2F showed the greatest degree of polarization, and consequently the best signal to noise. It was chosen as the label of choice for a primary screen. Data were fitted to a one site binding model using Graphpad prism.

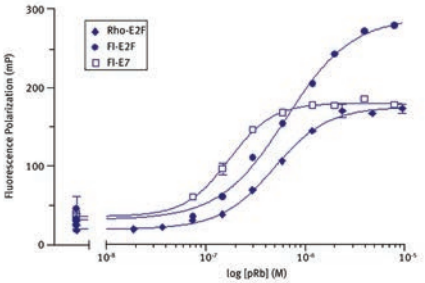

Fig. 2: Binding of fluorescein-E2F, rhodamine-E2F and fluorescein-E7 to pRb.

Kd values of (450±70) nM and (380±50) nM were calculated for fluorescein and rhodamine labelled E2F, which were similar to Kd determined for unlabelled peptide using isothermal calorimetry. Fluorescein-E7 showed tightest binding with Kd = (130±20) nM.
The assay was optimised in 384-well black plates (Matrix) and automated using a Beckman Fx liquid handling robot. 1 µM pRb in 50 mM Tris HCl, pH 7.0, 100 mM NaCl, 10 mM DTT, 0.05% NP-40 was mixed with 40 µM compound (4% DMSO) and 0.4 µM fluorescein-E2F (final concentrations). Total reaction volume 50 µL. Controls from a test screen of 10,000 compounds are shown in figure 3.

BMG LABTECH microplate reader settings

Fluorescein detection:	ex 485-12, em 520-30 nm
Rhodamine detection:	ex 545-7, em 580-12 nm
Default general settings:	50 flashes, 1.0 s positioning delay, 1 cycle

Gains were set using free fluoro-tagged peptide to give mP=35 and k=1.

Results & Discussion

Polarized and depolarized signal from fluorescein-E2F with and without pRb present are shown in figure 3 (solid and open blue circles respectively). Specific disruption of binding by E2F protein and peptide are also shown.

Rev. 07/2004
Keywords: **Binding studies, E2F peptide, False positives, Hit confirmation, Protein-protein interaction**

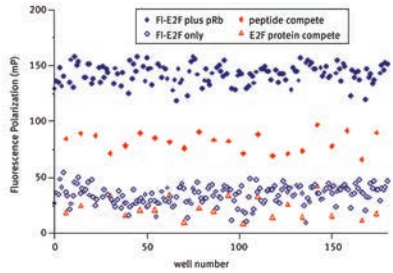

Fig. 3: Controls from a test screen of 10,000 compounds.

Addition of E2F protein completely displaces Fl-E2F (open red triangle) and the signal is reduced to that of free fluoro-peptide alone. Addition of unlabelled-E2F peptide (solid red diamond) at a concentration which gave 50% inhibition is clearly separated from the control populations. Hits were identified as compounds which reduced the polarization signal to less than mean-3sd of the fluorescein-E2F: pRb control. A summary of the screen data is shown below in table 1.

Table 1: Summary of screen data.

Assay Parameters	Signal: Noise Signal: Background Z'	6.9 4.8 0.67
Test Screen 10,000	Z' Hit Rate	0.45 0.93%

A large proportion (37.5%) of the hits selected from the primary screen were coloured compounds which significantly altered the fluorescence intensity signal, and were potentially interfering with the assay. All hits were included in the hit confirmation assays.

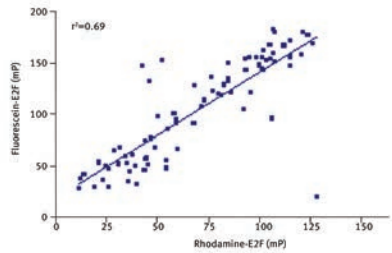

Fig. 4: Identification of fluorescence interfering compounds and correlation between inhibition of rhodamine-E2F and fluorescein-E2F.

Hits were replated from master stocks and retested against fluorescein-E2F and rhodamine-E2F. A correlation (r2=0.69) between inhibition of fluorescein-E2F and rhodamine-E2F was observed (figure 4) with a hit confirmation rate of 78%. Notably, 60% of compounds which were potentially interfering with the fluorescein signal were inhibitors with rhodamine-E2F assay, without affecting rhodamine fluorescence intensity signal. Suggesting that deselection of compounds on the basis of fluorescence interference can lead to loss of real inhibitors.

Finally the hits were tested against a second peptide binding site, fluorescein-E7 peptide at 400 nM, as shown in figure 5. The results were compared to inhibition of E2F and a scatter plot is shown.

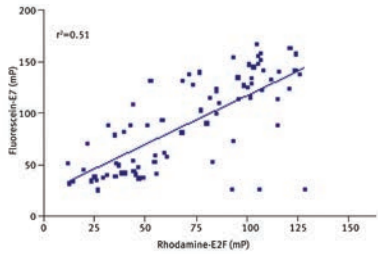

Fig. 5: Correlation between inhibition of fluorescein-E2F and fluorescein-E7 and identification of non-specific hits.

A weak correlation was observed (r^2=0.51), with 72% of the inhibitors of E2F also inhibiting fluorescein-E7. These compounds were excluded as non-specific inhibitors and were not taken forward in subsequent biochemical assays. 14 hits were taken forward from a total of 80 hits identified in the primary screen.

Conclusion

Binding of fluorescein-E2F to pRb showed a greater degree of polarization compared to binding of rhodamine-E2F, and consequently a better signal to noise ratio and z-factor could be obtained. It was the label of choice for a primary screen.

Screening of the hits against the second peptide site, E7, identified non-specific inhibitors, which caused gross structural changes to the protein. The combination of rhodamine-E2F and fluorescein-E7 with pRb in the same well allowed rapid selection of specific inhibitors, and minimised reagent usage. Identification of these non-specific inhibitors dramatically reduced the down stream work load.

PHERAstar® *FS*: a multi-mode microplate reader for sophisticated HTS assays

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- **Far Red PolarScreen™ Kinase Assay**
- **AlphaScreen cAMP competition assay**
- **HTRF® TNFα immunoassay**

Introduction

The High-Throughput Screening (HTS) approach to drug discovery has gained widespread popularity over the last 15 years. Because of the need to process thousands of assays per day, HTS relied upon multiple-well microplates and robotic processing technologies. Pressure for increase in throughput and reduction in cost led to the adoption of high-density, lower-volume microplates as well as fast, homogeneous, miniaturizable screening assays.

BMG LABTECH's PHERAstar® *FS* combines rapid plate reading necessary for HTS with enhanced performance and sensitivity needed to read small liquid volumes. The performance and features of the PHERAstar *FS* are presented in this application note with commercially available HTS kits such as Far Red PolarScreen™, AlphaScreen®, and HTRF®.

Materials & Methods

Far Red PolarScreen™ Kinase Assay

In fluorescence polarization mode, the Far Red PolarScreen™ assay (Life Technologies) for tyrosine kinase (Csk) was used in low volume 384-well plates (from Corning) with a final assay volume of 20 µL according to the kit protocol. The Far Red fluorophore was excited with 200 flashes at 610 nm and emission was detected in both polarization planes simultaneously at 670 nm.

AlphaScreen® cAMP competition assay

The cAMP assay kit (PerkinElmer) was performed in AlphaScreen® mode in accordance with the kit protocol in white 384-well small volume (SV) plates (Greiner Bio-One) with a final assay volume of 25 µL. The AlphaScreen® beads were excited with 400 flashes at 680 nm and emission was detected simultaneously in the range of 520 nm to 620 nm. To avoid evaporation, all plates were sealed with transparent microplate sealers during incubation or storage and the sealer was not removed during reading of the microplate.

HTRF® TNFα immunoassay

The time-resolved fluorescence mode was explored using the HTRF® reader control kit (Cisbio) that is designed for the calibration of HTRF® compatible readers. Results were obtained in, 384-well small volume plates (Greiner) according to the kit protocol. The incubation took place overnight at room temperature and the final assay volumes were 20 µL. The kit is based on a Tumor Necrosis Factor alpha (TNFα) assay and may also be used for reader validations. On the flash lamp-based PHERAstar *FS*, the assays were run with 200 or 400 flashes per well. The FRET donor europium cryptate was excited at 337 nm and emission was simultaneously read at 620 nm and 665 nm.

Results & Discussion

Far Red PolarScreen™ Kinase Assay

The Far Red PolarScreen™ assays employ a proprietary Far Red fluorophore in homogeneous fluorescence polarization assays.

Protein kinases (PKs) are a diverse group of enzymes involved in many areas of cell signalling. These include cell growth and proliferation and neural functions. The keen interest in PKs arises from their role in regulating biological mechanisms. Through phosphorylation, PKs participate in many cellular signal transduction processes. Research focused on kinase activity could ultimately identify targets that can be used to develop new pharmaceutical agents to treat many of these diseases.

In a Far Red kinase assay, kinase, substrate, and ATP are allowed to react in the presence of library compounds. After the reaction is complete, antibody and Far Red labelled tracer are added. The antibody can associate with either the labelled tracer (resulting in a high FP value) or the kinase-produced phosphorylated substrate (resulting in a lower FP value). The amount of antibody that binds to the tracer is inversely related to the amount of phosphorylated product present, and in this manner, kinase activity can be detected and measured by a decrease in FP value. Thus, library compounds that inhibit the reaction are identified as wells that have a high polarization value.

To demonstrate the use of the PHERAstar *FS* with Invitrogen's Far Red PolarScreen™ kinase assay, a tyrosine kinase (Csk) titration curve (n = 3) was performed, in the concentration range of 0.5 pg/mL to 2 µg/mL Csk, as shown in Fig. 1.

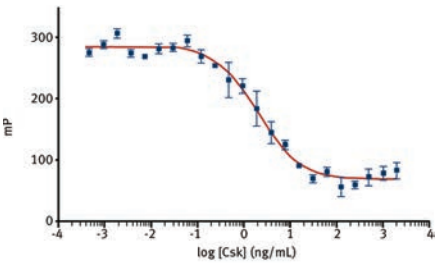

Fig. 1: Tyrosine kinase (Csk) titration with the PolarScreen™ Far Red detection kit on the PHERAstar *FS*.

AlphaScreen® cAMP competition assay

Cyclic AMP (cAMP) is one of the most important intra-

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Keywords: **1536-well, cAMP, Far Red PolarScreen, HTS, Simultaneous Dual Emission SDE, TNFα**

cellular mediators. Detection of cAMP with AlphaScreen® is based on the competition between cAMP produced by cells and a biotinylated cAMP probe that is sandwiched by streptavidin-donor and anti-cAMP antibody conjugated acceptor beads. A decrease in signal is observed with an increase in intracellular cAMP produced. In the absence of intracellular cAMP, a maximum signal is detected. To demonstrate the functionality of the AlphaScreen® assays and the performance of the PHERAstar *FS*, a titration curve (n = 3) with cAMP was performed (Fig. 2).

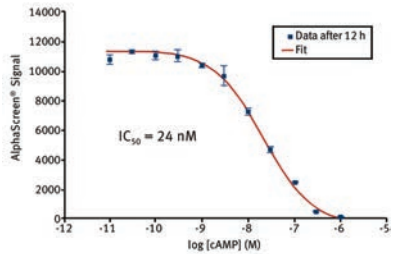

Fig. 2: A typical cAMP AlphaScreen® titration curve measured on the PHERAstar *FS*.

Final assay volume was 25 µL per well in a 384-well SV plate and after 12 hours incubation at room temperature the plate was read on PHERAstar *FS* using an integration time of 0.3 seconds per well. The concentration of cAMP was in the range of 1 µM to 10 pM and the cAMP titration curve reveals a high S/B = 82 value. MARS evaluation software, including a 4-parameter-fit function, was used for curve fit and IC50 determination. The calculated IC50 = 24 nM value complies with the literature. These experimental evaluations show that the PHERAstar *FS* can produce high quality data for the AlphaScreen® assay technology.

HTRF® TNFα immunoassay

Cisbio's HTRF® assays employ fluorescent Eu^{3+} cryptates (donor) and XL665 (acceptor) in homogeneous time-resolved FRET-based assays.

Tumor Necrosis Factor alpha (TNFα), a 17 kDa cytokine, is an important mediator secreted by activated macrophages and monocytes with a large spectrum of antiviral immunoregulation, metabolic and inflammatory properties. The HTRF® TNFα assay is a single step double-site immunometric assay involving two MABs conjugated either with europium cryptate or to XL665. The HTRF® TNFα assay principle is shown in Fig. 3.

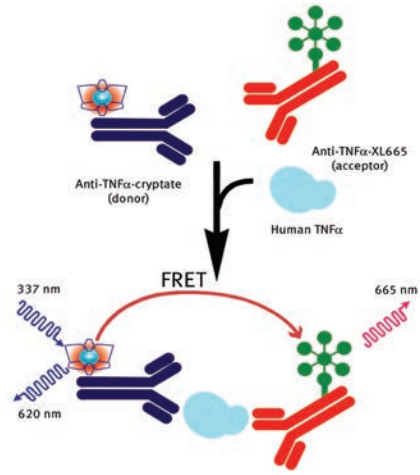

Fig. 3: Assay principle of the homogeneous time-resolved fluorescence immunoassay for TNFα. The assay is run using a straightforward "mix & measure" detection.

Under its final configuration, free TNFα from calibrators or samples is sandwiched by mouse MAb IPM2-Eu cryptate (IPM2-K) and mouse MAb IPM3-XL665 (IPM3-XL665) conjugates. The FRET signal generated by the simultaneous binding of the two conjugates is proportional to the amount of TNFα present in the sample. Both 665 nm and 620 nm signals were measured simultaneously on the PHERAstar.

Delta F is a value calculated from the 665 nm / 620 nm ratios which enables the data to be normalized with respect to between-assay variations. In addition, delta F is reader independent and can be used for indicating and comparing the quality of a reader. The PHERAstar *FS* results in an excellent delta F value (> 1100 % High calibrator).

Conclusion

The discovery of new leads through HTS is based on the ability to precisely measure biomolecular interactions and find successful detection strategies that are compatible with miniaturized HTS. The PHERAstar *FS* multimode reader proved to have a wide range of possible applications for HTS needs.

HitHunter® IP3 assay for GPCR screening using the PHERAstar® *FS*

Lindy Kauffman and Sherrylyn De La Llera
DiscoverX Corporation, USA

- **PHERAstar® *FS* multimode high-throughput microplate reader was used to access Gq coupled GPCRs**
- **HitHunter® is a robust and sensitive homogeneous fluorescence polarization assay to measure IP3 levels**
- **Assay miniaturization up to 1536-well plate format**

Introduction

G protein coupled receptor (GPCR) activation regulates cell signaling via several second messengers, including 3'-5'-cyclic AMP (cAMP), inositol phospholipids and calcium. The quantitation of accumulation of these second messengers is used to pharmacologically characterize both the action of GPCR ligands and to identify novel compounds in high-throughput screening (HTS). GPCRs coupling to $G\alpha_s$ and $G\alpha_i$ proteins activate or inhibit, respectively, adenylate cyclase, subsequently changing intracellular cAMP levels. GPCRs coupling to $G\alpha_q$ or $G\alpha_o$ proteins activates phosphoinositol phospholipase C-ß, an enzyme that hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) to form *sn*-1,2-diacylglycerol and inositol- 1,4,5-trisphosphate (IP3). IP3 binds and opens endoplasmic IP3 gated calcium channel, resulting in the release of bound calcium into the cytosol. Metabolic products of IP3 also modulate cell signaling, such as inositol 1,3,4,5-tetrakis-phosphate (Ins P4), which synergistically facilitates IP3, mediated calcium release. DiscoverX has developed a homogeneous assay based on fluorescence polarization (FP), to measure IP3 levels generated by GPCR activation. This application note, describes the use of IP3 assay along with PHERAstar *FS* multimode HTS plate reader.

The assay is based on FP mode using a proprietary binding protein that is highly selective for the active isomer of IP3. The assay is a competitive binding assay (figure 1), in which cellular IP3 displaces a fluorescent derivative of IP3 from a specific binding protein. The assay measures changes in FP, a single wavelength ratiometric technique, in which a fluorescent derivative of IP3 is used as a tracer. In the assay unlabelled IP3, either a standard IP3 solution, or derived from the cell lysate, displaces the tracer from the binding protein and the rotation time increases and low FP signal is measured. By this means, a calibration is generated to the standard IP3 dilutions and the molar concentration of IP3 in the cell lysate is determined by interpolation.

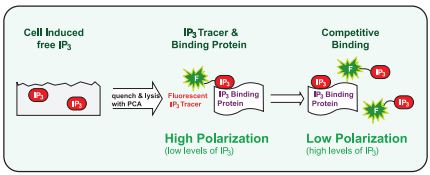

Fig. 1: HitHunter® IP3 fluorescence polarization assay principle.

Materials & Methods

BMG LABTECH's PHERAstar *FS* combines rapid plate reading necessary for HTS with the enhanced performance and sensitivity needed to read small fluid volumes. The multimode microplate reader has the flexibility to excel with the most demanding assays and is designed to read all leading HTS detection modes (fluorescence intensity, time-resolved fluorescence, fluorescence polarization, luminescence and absorption) in all formats up to 1536. The PHERAstar *FS* was run in FP mode for the monitoring of the HitHunter® IP3 demo kit (DiscoverX Corp.) containing the following reagents:

HitHunter IP3 kit reagents	
1	IP3 Standard – 20 µM
2	IP3 Standard Dilution Buffer
3	Perchloric Acid (PCA) – 0.2 N
4	IP3 Tracer - Green
5	IP3 Binding Protein

The standard curve for DiscoverX's IP3 kit was run according to the package insert protocol in black 384-well plates (non-binding polypropylene plates; Greiner), and the fluorescence polarization signal was read on the PHERAstar *FS* one hour after the addition of the last reagent using the following protocol for plate reader setup (figure 2).

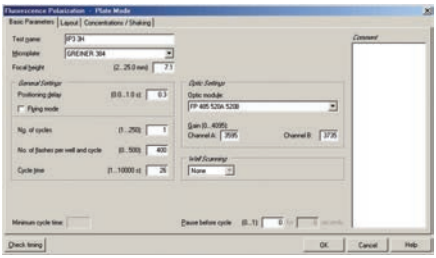

Fig. 2: This screenshot shows the HitHunter® IP3 FP assay setup window from the PHERAstar *FS*. An optical module for HitHunter® IP3 is directly available from BMG LABTECH.

The table below outlines the volumes and procedures for the IP3 protocol measuring both standard curve and agonist/antagonist treated cells in a 384-well plate format. Measuring agonist/antagonist treated cells, IP3 levels were stabilized with the addition of 5 µL of 0.2 N perchloric acid.

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Keywords: **1536-well, Competitive binding, GPCR, HitHunter, IP3**

Table 1: HitHunter® IP3 protocol for standard curve and agonist/ antagonist treated cells.

Hit Hunter IP3 Protocol			
Condition	**Standard Curve**	**Cells Agonist**	**Cells Antagonist**
Step 1: Standard/ cells	10 µL standard	10 µL cells	10 µL cells
Step 2: Antagonist	-	-	5 µL antagonist
Incubate	-		30 min. at 37° C
Step 3: Agonist	5 µL water	5 µL agonist	5 µL agonist
Incubate	-	20 seconds (room temperature)	
Step 4: PCA	5 µL PCA -0.2N		
Step 5: Tracer	10 µL IP3 Tracer-Green		
Step 6: Binding Protein	20 µL IP3 Binding Protein		
Gently tap plates for even mixing (shake plates for 5 minutes)			
Read fluorescence polarization signal on the PHERAstar *FS* (Excitation filter 485 nm/Emission filter 520 nm)			

Results & Discussion

DiscoverX Hithunter® IP3 was prepared in 384-well format and standard curves were run on BMG LABTECH's PHERAstar *FS* in fluorescence polarization mode. IP3 standard reagents were added according to the assay protocol and the fluorescence polarization signal was read one hour after the addition of the last reagent. The Hithunter® IP3 standard curve is illustrated in the figure below (figure 3).

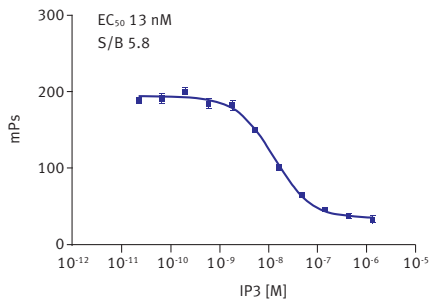

Fig. 3: Hithunter® IP3 Assay - Green: Standard curve data in 384-well format.

The Hithunter® IP3 assay is a robust, sensitive and specific tool for measuring cellular D-myo-inositol 1,4,5-triphosphate. The signal is based on competitive binding between an IP3 fluorescent tracer and an unlabeled IP3 from cell lysate or IP3 standard. The signal is read as a change in fluorescence polarization and is inversely proportional to the amount of IP3 in cell lysates. The standard curve signal to background is >5, and the EC_{50} is 13 nM. The signal can be measured immediately or up to 16 hours later.

Conclusion

GPCRs are critical targets in HTS drug discovery and GPCR signaling can be examined by direct quantitation of IP3 by applying the HitHunter® IP3 kit. Good results were obtained on the PHERAstar *FS* multimode microplate reader which is designed to read all leading HTS detection modes in formats up to 1536. The high degree of sensitivity, easy-to-use software, robust hardware and optimized detection systems make the PHERAstar *FS* ideal for GPCR analyses in the high-throughput assay environment.

The PHERAstar was run in FP mode for the HitHunter® IP3 assay, which uses FP for sensitivite detection and ease of use. The assay is ideal for the detection of basal levels of IP3 in cell lysates, as well as low levels for IP3 induction. Once the cells are lysed, the stable assay signal can be read the same day or overnight. There are few robust IP3 assays currently available and none easily scalable to 1536 automation. DiscoverX offers this assay to give maximum precision and reliability for GPCR screening.

For more information on DiscoverX assays please refer to the web site: www.discoverx.com

Predictor™ hERG fluorescence polarization assay kit performed on the PHERAstar® *FS*

E.J. Dell
BMG LABTECH, Durham, NC USA

- **Predictive – generates IC50 values that are tightly correlated with those from patch-clamp electrophysiology**
- **Nonradioactive – eliminates radioligands by utilizing a fluorescence polarization tracer**
- **Add and read – enables high-throughput hERG liability screening in a homogenous assay format**

Introduction

The potential for cardiotoxic side-effects continues to challenge the development of small molecule based therapies. These intolerable side-effects are often precipitated by drug-induced long QT syndrome (LQT), which is often linked to blocking the human ether-a-go-go related gene (hERG) potassium channel. Although patch-clamp electrophysiology remains the gold standard for determining the interaction of compounds with the function of the hERG channel, radioligand displacement assays have proven to be a cost-effective initial alternative assay for hERG channel liability at early stages of compound development. Speed and cost still suffer however, as radioligand displacement assays remain heterogeneous and depend on the procurement and disposal costs of radioligands. Because the FDA has recommended that all new drug candidates be tested for blockage of the hERG channel, there is a pressing need for a simple, safe, cost-effective method to triage compounds earlier in the discovery process.
To address this need, the Predictor™ hERG Fluorescence Polarization Assay Kit from Invitrogen has been developed. The assay is based upon the principle of fluorescence polarization (FP), in which a red-shifted fluorescent tracer is displaced from the hERG channel by compounds that bind to the channel. When the tracer is bound to the channel the FP value is high, and when displaced by hERG binding compounds the FP value is low (Figure 1).

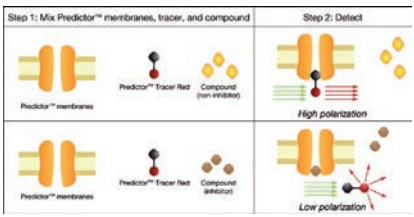

Fig. 1: Schematic of the Predictor™ hERG Fluorescence Polarization Assay.

The assay kit includes a stable membrane preparation that contains high levels of hERG protein, as well as optimized tracer, buffer, and controls necessary to perform the assay. This technical note describes the performance of the Predictor™ hERG Assay on the BMG LABTECH's PHERAstar® *FS* multifunctional HTS microplate reader.
BMG LABTECH's PHERAstar *FS* is a multidetection microplate reader that combines rapid plate reading necessary for high throughput screening (HTS) with enhanced performance and sensitivity needed to read small fluid volumes. The PHERAstar *FS* has been designed to read all HTS detection modes (fluorescence intensity, time-resolved fluorescence, fluorescence polarization, luminescence, and absorption) in all plate formats up to 1536 wells. The PHERAstar *FS* uses a unique application-specific optic module in conjunction with a reading head featuring five photomultiplier tubes that can simultaneously measure two emission signals at any desired wavelength. This Simultaneous Dual Emission (SDE) detection on the PHERAstar *FS* provides for outstanding sensitivity and accuracy in fluorescence polarization assays, minimizing read times and increasing Z' values.

Materials & Methods

- PHERAstar *FS* microplate reader with Predictor™ FP module (540-20/590-20), BMG LABTECH
- Black 384 well low volume microplates from Corning
- Predictor™ hERG Fluorescence Polarization Assay Kit from Invitrogen

Follow the Predictor™ hERG Fluorescence Polarization Assay Kit instructions. Briefly, a 10 µL dilution series of the compound was placed in a 384 well plate (in duplicate), followed by 5 µL additions of both tracer and hERG membrane preparations. The plate could then be read any time between a 1 and 24 hour (or more) incubation.

Results & Discussion

Competitive Binding of Known hERG Blockers

Ten compounds that have been previously shown to block the hERG channel were tested in the Predictor™ hERG Fluorescence Polarization Assay using the BMG LABTECH PHERAstar *FS* (Fig. 2).

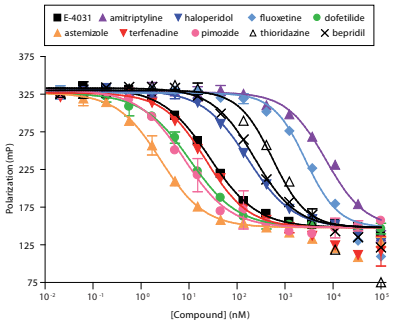

Fig. 2: Competitive binding assay of 10 known hERG blockers using the Predictor™ hERG Fluorescence Polarization Assay.

Rev. 12/2008
Keywords: **Cardiotoxicity, E-4031, hERG, Ion channel, Patch-clamp**

The polarization data are shown in Figure 2, with the data summarized and compared with patch-clamp or radioligand displacement data that have been previously reported in Table 1. Non-specific displacement of the tracer from the membrane in the FP assay (observed with some compounds at high concentrations) was corrected by fixing the baseline of the displacement curve to the value seen with saturating levels (30 µM) of E-4031, a compound for which no non-specific displacement of tracer was observed.

IC_{50} [nM]			
Compound	Predictor™ FP	Patch Clamp	Radioligand
E-4031	27	48	20
amitriptyline	7118	10000	2440
haloperidol	167	174	90
fluoxetine	2687	990	2230
dofetilide	11	12	40
astemizole	3	1.2	1
terfenadine	23	16	30
pimozide	8	18	6
thioridazine	553	1250	510
bepridil	230	550	170

Determination of Assay Z' Value

Assay robustness was assessed by measuring control wells containing either 30 µM E-4031 (to completely displace tracer from binding to hERG) or no compound. All wells contained 1 % DMSO. The data are shown in Figure 3 and a Z' value of 0.86 was determined. Although not shown here, the assay is stable at DMSO, methanol, or ethanol concentrations up to 5 %.

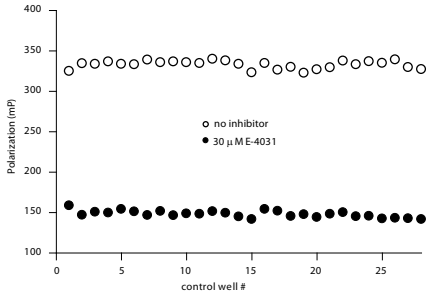

Fig. 3: Z' Control Wells for the Predictor™ hERG Fluorescence Polarization Assay performed on BMG LABTECH's PHERAstar *FS*.

Conclusion

Because of the potentially lethal effects of unintended hERG block, the FDA has recommended that all compounds be functionally tested for this effect in an electrophysiological (patch-clamp) assay before being tested in humans. Due to the need for highly specialized equipment and expertise, this test is typically performed only at the later stages of the discovery process. While assessment of hERG channel function will remain the gold standard to assess hERG channel block, binding assays can be used in order to triage compounds and identify potential liabilities earlier in the process. The recently developed Predictor™ hERG Fluorescence Polarization Assay Kit provides a homogenous assay format that avoids the problems of using radioisotopes to meet this need. In addition, the PHERAstar *FS* multifunctional HTS microplate reader provides one of the fastest and most sensitive platforms to run this FP assay.

PHERAstar® *FSX*
PHERAstar® *FS*

CLARIOstar®

High-throughput protein-DNA affinity measurements using fluorescence anisotropy

Dmitry B. Veprintsev
MRC Laboratory of Molecular Biology, Cambridge, CB2 0QH, UK

- **Fluorescence anisotropy titrations used to quantify p53-DNA interactions**
- **96 titration experiments performed in parallel**
- **Applicable to a wide range of applications**

Introduction

The tumour suppressor protein p53 is a transcription factor that plays a key role in response to carcinogenic stress and prevention of tumor development. Mutations in p53 are associated with 50% of all human cancers. p53 recognises a 20 bp DNA sequence consisting of two repeats of 5'-RRRCWWGYYY-3' (where R=A or G; Y=C or T; W=A or T), separated by 0-13 bp. The genomic DNA in the cell often contains modified nucleotides, such as 5-methylcytosine. Using fluorescence anisotropy titrations, we studied the effect of modified nucleotides on the recognition of DNA by the tumour supressor p53 (Figure 1).

Assay Principle

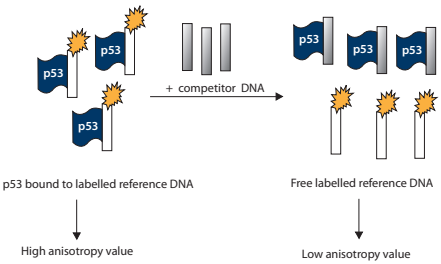

Fig. 1: Assay principle for anisotropy measurements.

p53 binds to a labelled reference DNA resulting in a high anisotropy value. After adding of competitor DNA, the reference and competitor DNA will compete for the binding sites of p53. The competitor DNA will substitute the reference DNA depending on the affinity of p53 for it and depending of its concentration resulting in a lower anisotropy value.

Fluorescence anisotropy is the property of fluorescent molecules to retain the polarisation of the excitation light and reflects the tumbling rate of molecules in solution. It is ideal for studying protein- DNA interactions as the complex formed is larger and tumbles more slowly than the unbound oligonucleotide.
Here, we describe an experimental setup where the whole titration experiment is done in a single well. Such setup significantly improved data quality and allows full utilisation of the PHERAstar® *FS* HTS microplate reader from BMG LABTECH.

Materials & Methods

- Black 96-well microplates from Corning
- Oligonucleotides containing modified bases (competitor DNA) and Alexa488-labelled consensus oligonucleotide (reference DNA) from Eurogentec
- epMotion pipetting robot from Eppendorf
- Bravo liquid handling robot from Velocity 11
- PHERAstar *FS* from BMG LABTECH

The super-stable mutant of full length p53 was used. Oligonucleotide concentrations were measured and normalized to 1 mM prior to annealing. Oligonucleotides were annealed by heating to 95°C for 5 min and cooling at 1°C/min to room temperature and diluted to a final concentration of 50 µM.
DNA binding experiments were done by fluorescence anisotropy at room temperature (25°C). The buffer conditions for the binding experiments were 25 mM phosphate buffer pH 7.2, 225 mM NaCl, 10 % v/v glycerol and 5 mM DTT. Total ionic strength was 286 mM. Bovine serum albumin (BSA, 0.2 mg/mL) was added to buffers to minimise non-specific binding of proteins. The concentration of labelled reference DNA was 20 nM. For measurements of the binding affinity of the reference DNA (direct titration), a p53 stock solution of 1.25 µM also contained 20 nM reference DNA so that the concentration of reference DNA was kept constant during the experiment. For competition experiments, 20 nM reporter DNA was mixed with full length p53 to a final concentration of 125 nM. A 25, 50 or 100 µM stock solution of competitor DNA was added in small aliquots to compete the reference DNA off the complex. In order to keep the concentration of reference DNA and p53 constant, the competitor DNA stock solution also contained labelled reference DNA and p53. Thus, only the concentration of the competitor DNA changes during titration. The DNA-binding experiments were multiplexed by performing titrations in black 96-well plates with a Bravo 96-channel pipetting robot interfaced with a PHERAstar *FS* microplate reader with a 485/520 nm fluorescence polarisation module. The detection mode settings were optimised for the focus position 2 mm below the maximum signal height. The sample volume during the titration was kept constant (200 µL): prior to addition of an aliquot of the binding protein (direct titration) or competitor DNA (competition experiments), an equal volume of sample was removed by aspiration. Each competitor DNA sequence was represented at least 3 times per plate. Each plate contained the reference sequence as a control.

Rev. 09/2008
Keywords: **Alexa488, Anisotropy, p53, Protein-DNA interaction, Transcription factor**

The anisotropy values are automatically calculated using the following formula:

$$R = \frac{I_a - I_b}{I_a + 2I_b}$$

whereas I_a is the parallel emission light and I_b is the perpendicular emission light.

Results & Discussion

The 22 bp palindromic reference sequence CGGACATGTCCGGACATGTCCG, consisting of representative p53 consensus sequence flanked by a single C or G nucleotide, was labelled at the 5´end with Alexa488 fluorophore. The results are presented in Figure 2.

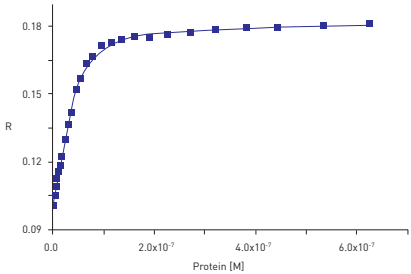

Fig. 2: 20 nM of Alexa488-labelled oligonucleotide was titrated by increasing concentration of p53. The formation of the protein-DNA complex is reflected by an increase in the observed anisotropy values (squares).

The analysis of the binding data according to a Hill equation (Figure 2, solid line) allows determination of Kd of interaction (Kd = dissociation constant of p53 from DNA sequence).

In subsequent experiments, we used a competition assay to improve the accuracy of determination of the difference in the affinity of the binding protein for the reference and competitor DNA (Figure 3). Analysis of the competition curve allowed accurate determination of the difference in affinity of the two nucleotides.

The results of the competition assay (an example is given in Figure 3) allow to determine the difference of Kd between two sequences. Such titrations were done for every DNA sequence studied.

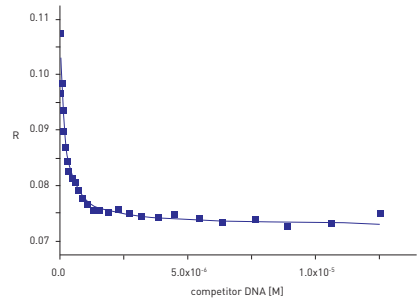

Fig. 3: A displacement of the Alexa488-labelled reference oligonucleotide from the complex by an unlabelled competitor oligonucleotide results in decreasing anisotropy values (R).

The binding affinity of a specific DNA sequence depended on the exact postion and nature nucleotide modification (Figure 4). The variations in the affinity were as much as one order of magnitude.

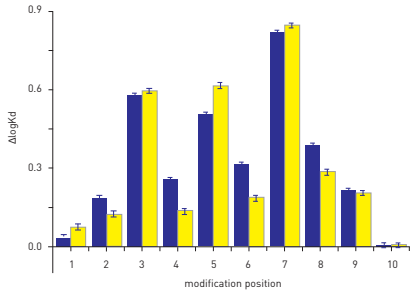

Fig. 4: Effects of dinucleotide modifications on affinity of p53 for DNA. Nonmodified (blue) or modified (yellow) dinucleotide was systematically substituted into the reference sequence at positions 1-10 of the first half site.

The difference in affinity relative to the unmodified reference sequence is shown by the ΔlogKd in Figure 4. The logKd of the reference sequence was -7.55. As an example, introduction of modified dinucleotides at positions 4 and 6 have a small overall impact on the affinity of p53 and result in the biggest modification specific response.

Conclusion

The PHERAstar *FS* from BMG LABTECH, interfaced with a liquid handling instrument, provides a powerful multiplexing platform for high throughput determination of binding affinities using fluorescence anisotropy. The sensitivity of the PHERAstar *FS* in the fluorescence polarisation mode allowed us to collect data suitable to accurate determination of Kd.

Membrane fluidity measurements using UV fluorescence polarization

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- **Membrane fluidity is measured in both liposomes and mitochondrial membranes via fluorescence polarization**
- **DPH (1,6-diphenyl-hexa-1,3,5-triene) used as fluorescent dye for FP measurements**
- **The effects of dibucaine and propranolol on membrane fluidity are measured**

Introduction

Membrane fluidity and other properties of lipids are commonly assessed using various fluorogenic membrane probes and fluorescence polarization (FP) measurements. The basic principle is that alterations in lipid packing (e.g. temperature-dependent lipid phase transitions) change the mobility of a membrane-bound fluorophore. The latter parameter (specifically, "rotational relaxation") can be monitored by exciting the fluorophore with a polarized light and measuring the emitted light in two planes - parallel and perpendicular to the polarization plane of the excitation light. FP is defined as the following ratio: $I_{||} - I_{\perp} / I_{||} + I_{\perp}$, where $I_{||}$ and $I_{\perp}$ are fluorescent intensities measured in the parallel and perpendicular channels, respectively.

Membrane fluidization increases mobility of the dye and decreases the intensity of the emitted parallel component. Accordingly, FP is reciprocally related to the membrane fluidity. Optical characteristics of the fluorescent dye DPH strongly depend on the environment; the dye is almost non-fluorescent in aqueous solutions, while binding to the hydrophobic region of the membrane results in a sharp increase in the fluorescence signal (with an excitation maximum in UV range). This note describes the use of a BMG LABTECH microplate reader for measurements of relative changes in lipid fluidity of DPH-labeled membranes.

Materials & Methods

- Phospholipids from Avanti Polar Lipids
- Mitochondria isolated from rat liver
- Opaque-walled, clear bottom 96-well plates from Costar
- Microplate reader from BMG LABTECH, equipped with UV FP optics

Membrane fluidity measurements were performed in liposomes and isolated mitochondria. 100 µl of samples were placed in a microplate. A 355 nm filter is used for excitation. Emission is measured at 430 nm.

To validate the method, FP measurements were taken using liposomes with defined gel-to-liquid crystalline phase transition temperature. Unilamellar liposomes were prepared from 1-myristoyl-2-palmitoylsn-glycero-3-phosphocholine (MPPC). The transition temperature for this lipid is in the 35 - 37°C range. For fluorescent labeling, MPPC liposomes were pre-incubated with 10 µM DPH (Sigma) at 45°C for 30 min in a KCl-based buffer (150 mM KCl; 10 mM HEPES, pH 7.4; 2 mM EGTA).

Steady-state FP measurements were taken at 25 - 45°C. Temperature on the microplate reader's incubator was increased by 2°C and FP measurements were taken after equilibrating the samples for 10-15 minutes. Data were acquired in endpoint mode.

A more complex model was investigated, native mitochondrial membranes. Isolated rat liver mitochondria (0.2 mg/ml) were preincubated with 10 µM DPH at room temperature for 40 minutes. Temperature in the microplate reader's incubator was increased by 2°C and the samples were equilibrated for 10 minutes at each given temperature before FP measurements were taken. Mitochondria used in these experiments were in a deenergized (non-respiring) state. Lastly, the effects of membrane active drugs dibucaine and propranolol on membrane fluidity in liposomes and mitochondria were determined.

Liposomes were formed from the following lipid mixture mimicking mitochondrial membranes: 47% phosphatidylcholine (PC), 28% phosphatidylethanolamine (PE), 9% phosphatidylinositol, 9% phosphatidylserine (PS), and 7% cardiolipin (CL). Data were acquired in kinetic mode.

Results & Discussion

As shown in Figure 1, increasing the temperature from 25 to 35°C did not change the FP values in MPPC liposomes. This reflects the expected high ordered gel state of the lipid in this temperature range. Increasing the temperature from 35 to 39°C results in a sharp, large-scale decrease in FP values corresponding to the phase transition. In the liquid phase, FP values continued to decline as the temperature increased to 45°C.

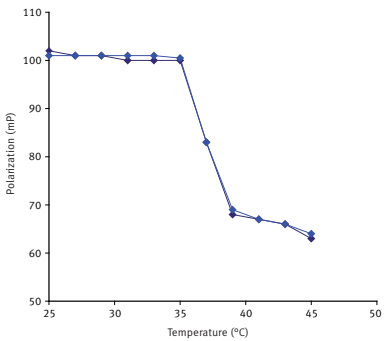

Fig. 1: Effect of temperature on FP measured in DPH-labeled MPPC liposomes. Target FP value was set to 100 mP for the DPH-labeled liposome sample equilibrated at 25 °C.

Rev. 12/2009
Keywords: **DPH, Fluidity, Liposomes, Membrane, Mitochondria**

Unlike MPPC vesicles, no rigid (temperature-insensitive) state or large-scale phase transitions were observed in the mitochondrial membranes (Fig. 2). A gradual decrease in FP in the 25 - 45°C temperature range is seen, which is consistent with previously published data on membrane fluidity measurements in DPH-labeled deenergized mitochondria. Relative FP values in whole mitochondria and purified outer mitochondrial membranes (OMM) were also compared. The patterns of temperature-dependence were similar in both membrane systems, but the relative FP values in OMM were significantly higher than those obtained in whole mitochondria (Fig. 2). This result can be readily explained by the higher cholesterol level in the OMM compared to the inner mitochondrial membrane.

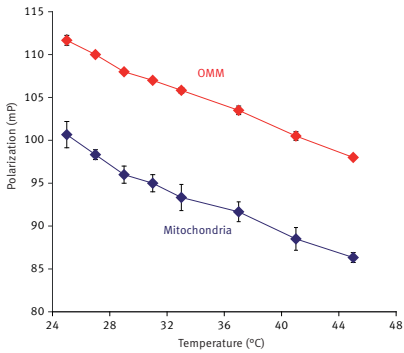

Fig. 2: Effect of temperature on FP measured in DPH-labeled isolated mitochondria and purified outer mitochondrial membranes (OMM). Target FP value was arbitrary set to 100 mP for the DPH-labeled mitochondrial sample equilibrated at 25°C. Data shown are average of 3 replicates.

Finally, the effects of propranolol and dibucaine on liposomes and isolated mitochondria were examined. Depending on concentrations, these drugs can increase membrane fluidity or rigidify some membranes. Figure 3 shows that the addition of propranolol to liposomes (panel A) results in a rapid decrease in relative FP values. A similar effect was observed in isolated mitochondria (panel B). The propranolol-induced decrease in FP was smaller in comparison to the dibucaine induced changes, but nonetheless was readily detected. The concentrations of the drugs (40-80 µM) were in the same range as those affecting mitochondrial activities. The data suggest that some of the effects of the drugs on mitochondrial function may be due to an increase in membrane fluidity.

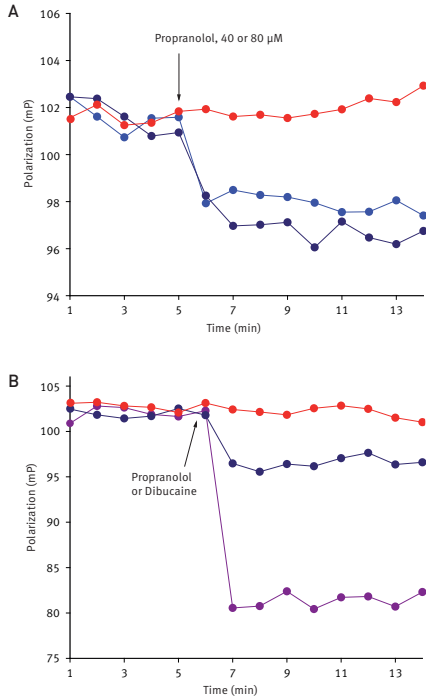

Fig. 3: Effect of propranolol and dibucaine on FP measured in DPH-labeled PC/PE/PS/PI/CL liposomes (A) and isolated mitochondria (B). Arrows indicate the addition of drug - 40 µM propranolol (light-blue line), 80 µM propranolol (dark- blue line), 80 µM dibucaine (purple line), control (red line). Red lines – control samples. Incubation conditions are the same as in Fig. 1 and 2.

Conclusion

This study demonstrates the suitability of the BMG LABTECH microplate reader with UV FP optics for measurements on membrane fluidity in a microplate-based format. The method allows for the monitoring of large scale alterations in membrane fluidity (such as phase transitions), as well as more subtle changes in lipid dynamics. This approach can be used as a high-throughput screening assay in the search for compounds altering lipid fluidity.

Methyltransferase, acetyltransferase, kinases, and GTPases can all be measured with Transcreener® assays

Meera Kumar[1], Tom Zielinski[1], Andrew Koop[1], Franka Ganske[2], and E. J. Dell[2]
[1] BellBrook Labs; [2] BMG LABTECH

- **The universal transcreener® assays measure the byproducts of common biological enzymatic reactions**
- **For epigenetic studies, AMP/GMP can be monitored for methyltranferase or acetyltransferase activity**
- **ADP monitored for kinases, ATPases, or helicases; GDP for G-proteins; and UDP for glycosyltransferases**

Introduction

Transcreener® assays from BellBrook Labs offer generic, universal HTS assays for nucleotide detection like ADP, UDP, GDP and AMP. These assays are available in three detection modes - Fluorescent Polarization (FP); Fluorescent Intensity (FI) and Time Resolved Fluorescence Resonance Energy Transfer (TR-FRET).

The PHERAstar series of microplate readers are perfect for all three detection formats. For FP and TR-FRET it uses a unique Simultaneous Dual Emission detection system that measures both emission wavelengths in one read of the microplate. Simultaneous Dual Emission detection not only reduces plate read times by half, it corrects for any signal variations due to differences in well volumes, concentrations, or fluctuations in excitation energy.

In this appication note we show the performance of PHERAstar *FS* for detection of different nucleotides in the Transcreener® FP assay. The instrument can achieve a Z' >0.8 and a Δ mP greater than 100 at 10% conversion for all the Transcreener® assays tested with 10 µM respective standard curve.

Assay Principle

Transcreener® Fluorescent Polarization Assay

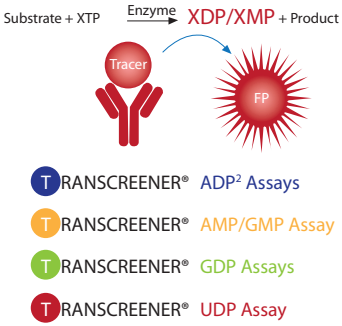

Fig. 1: Transcreener® Fluorescent polarization assays are competitive immunoassays that use a far red tracer bound to a selective antibody for ADP, UDP, GDP and AMP. The tracer is displaced by the above mentioned nucleotide products generated during enzyme reactions. The displaced tracer freely rotates leading to a decrease in fluorescence polarization, relative to the bound tracer.

Materials & Methods

- Transcreener XMP Detection Reagents
- Black 384-well plates from Corning
- PHERAstar® *FS* microplate reader (BMG LABTECH)

Representative for all XDP Transcreener assays the ADP² assay is explained in more detail. As the ratio of ADP:ATP increases, the proportion of bound tracer versus free tracer decreases, resulting in an overall decrease in mP values. A 15-point standard curve was prepared as described below:

1. Dispense 10 µL of each ATP/ADP combination across an entire row of the black 384-well plate.
2. Add 10 µL of ADP Detection Mix to those rows.
3. Dispense 10 µL of the 10 µl ATP/0 µM ADP combination into row P.
4. Dispense 10 µL of free tracer into wells P1 to P12.
5. Dispense 10 µL of buffer blank into well P13-P24.

For the FP measurement the Transcreener specific FP modul should be installed. The excitation wavelength is at 590 nm and both emission wavelengths are at 675 nm. A focus and Gain Adjustment should be done, while setting the target mP value to 20 mP using a well that contains only the free tracer. A detailed description of the procedure is given on BellBrookLabs application note: http://www.bellbrooklabs.com/PDFs/ApNote_ PHERAstar_FP.pdf

Results & Discussion

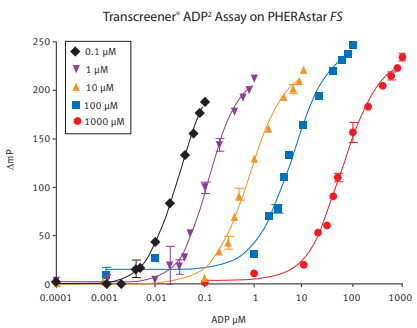

Fig. 2: Graph shows standard curves run on PHERAstar *FS* using 1000 µM, 100 µM, 10 µM, 1 µM and 0.1 µM initial concentrations of ATP using the Transcreener® ADP² assay.

Rev. 05/2012
Keywords: **ADP, AMP, GDP, Kinase, Transcreener, UDP**

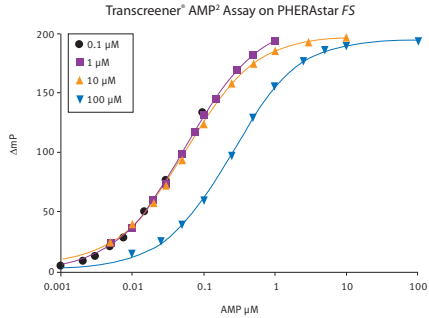

Fig. 3: Graph shows standard curves run on PHERAstar *FS* using 100 µM, 10 µM, 1 µM and 0.1 µM initial concentrations of ATP using the Transcreener® AMP² assay.

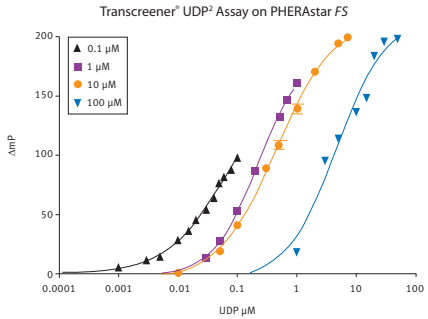

Fig. 4: Graph shows standard curves run on PHERAstar *FS* using 100 µM, 10 µM, 1 µM and 0.1 µM initial concentrations of UDP Glucuronic Acid using the Transcreener® UDP² assay.

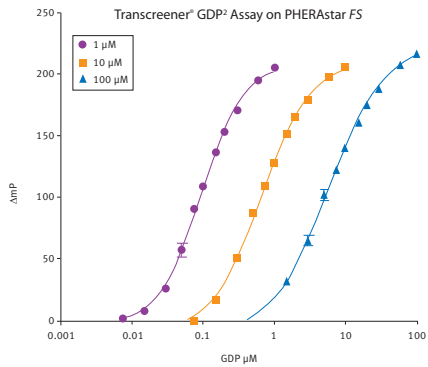

Fig. 5: Graph shows standard curves run on PHERAstar *FS* using 100 µM, 10 µM and 1 µM initial concentrations of GTP using the Transcreener® GDP assay.

Table 1: Table shows Z' values for 10 µM ATP/ADP; 10 µM ATP/AMP; 10 µM UDP Glucuronic Acid/UDP and 10 µM GTP/GDP generated in PHERAstar *FS*. The highlighted values show the Z' at 10% conversion demonstrating a superior assay performance in BMG LABTECH's PHERAstar *FS*.

% Conv	ADP² Assay	AMP² Assay	UDP² Assay	GDP Assay
20	0.88	0.92	0.86	0.86
10	0.85	0.93	0.91	0.86
5	0.82	0.93	0.85	0.81
4	0.77			
3	0.74	0.86	0.83	
2	0.58	0.55	0.57	0.67

Table 2: Read Times for the PHERAstar series of microplate readers in Fluorescence Polarization Mode (10 flashes, different microplate formats).

Fluorescent Polarization Read Times (10 flashes)		
	PHERAstar *FS*	Reader with No Simultaneous Dual Emission Detection
384-well	0:38	≥ 1:16 (0:38 x 2)
1536-well	1:52	≥ 3:44 (1:52 x 2)
3456-well	9:47	≥ 19:34 (9:47 x 2)

Conclusion

Bellbrook Labs offers four Transcreener assays for direct detection of ADP, AMP/GMP, UDP and GDP.
Excellent Z' values and large signals are shown for low percent conversion with all the four assays using BMG LABTECH`s PHERAstar *FS*.
This application note demonstrates the validation of the BMG LABTECH PHERAstar *FS* instrument for use with the Transcreener FP Assays. By utilizing the optimized instrument settings suggested within this Application Note, Z' values > 0.7 and ΔmP values > 120 at 10% conversion are achievable.

Protein-ligand binding measurements using fluorescence polarization

Valentina Speranzini[1], Alexander Fish[2] and Andrea Mattevi[1]
[1] Lab of Structural Biology, University of Pavia, Italy; [2] Netherlands Cancer Institute, Amsterdam, The Netherlands

- **Fluorescence polarization assay used to probe protein-ligand interactions**
- **Binding affinities are consistent with activity assays**
- **Technique sensitivity allows analysis of a wide range of concentrations**

Introduction

Human Lys-specific Histone Demethylase 1 (LSD1) promotes the demethylation of mono- or di-methyl-Lys4 on histone H31: as chromatin remodelling enzyme, LSD1 is found in complex with several partners, one of which being the REST Co-repressor 1 (CoREST1). We used the fluorescence polarization (FP) as a bio-physical tool to analyse the binding properties of LSD1-CoREST1 (LC1) hetero-dimer to H3-derived peptides (Fig. 1), that have been conjugated at the C-terminal with the fluorescent label TAMRA (5-Carboxytetramethylrhodamine).

Assay Principle

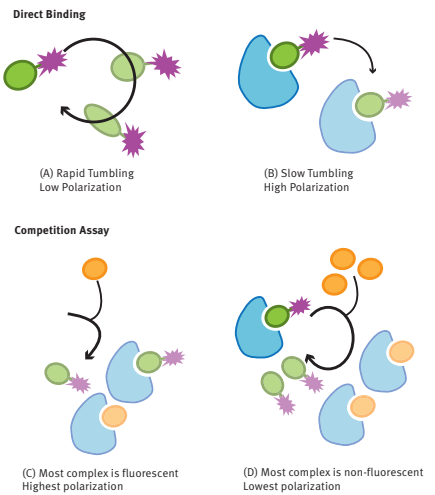

Fig. 1: Assay principle for monitoring protein-ligand interaction using fluorescence polarization.

(A) H3 labeled peptides in solution show a rapid rotation that causes dispersion in different direction of the incident polarized light: the resulting emitted polarization signal is low. (B) Upon binding to LC1, the high-molecular weight complex results in a slow rotation of the fluorescent molecule leading to high polarization values. (C,D) Non-tagged peptides, in orange, compete for LC1 active site. As competitor concentration increases, more freely labelled peptide remains in solution. This results in a low FP value, comparable to results in (A).

Materials & Methods

- black, 384-well, low flange microplates from Corning, UK
- TAMRA-conjugated peptides provided by NKI Protein Facility (Amsterdam)
- LC1 protein was recombinantly produced, according to reference 1
- Multi-mode microplate reader from BMG LABTECH, Germany

Experimental setup

1 nM labelled peptides were mixed to assay buffer: 15 mM KH_2PO_4 pH 7.2, 5% glycerol, 1 mg/ml BSA. Mix was then distributed equally across the plate. For each experiment, triplicates were prepared: starting concentration of protein LC1 was 4 µM, and then this was serially diluted 1:1 using the mix in the other wells. For competitive assays, constant enzyme concentration (around Kd) were mixed with 1 nM labelled ligand in the same assay buffer as above. Competitors were then titrated in using serial 1:1 dilutions as described for direct binding assay. The typical concentration range started from 10 µM.

CLARIOstar® instrument settings

Detection Mode:	Fluorescence Polarization, Endpoint
No. of flashes:	50
Excitation:	540-20
Emission:	590-20
Target temperature:	25°C

Focus and Gain adjustments for both channels were set on a reference well containing just labelled ligand. It was chosen to set the adjusted polarization to a reference value of 35 mP. Typical adjustment values for gain were between 1600 and 2000.

Results & Discussion

We first devised a direct binding experiment using three different H3-derived peptides, all previously described as substrates and/ or inhibitors of LC1 complex. On a relative scale, results obtained are consistent with activity measured before by the working group. The difference in the peptides consisted in different lengths and/or different amino-acid compositions as listed below:

meH3 (pep01) = H3 N-terminal tail, methylated on

Rev. 10/2014
Keywords: **Binding studies, Epigenetics, Histone, Protein-ligand interaction, TAMRA**

Lys4, 21 residues

H3K4M (pep02) = H3 N-terminal tail, baring the mutate residue in position 4, 21 residues

SN12 (pep03) = Transcription factor SNAIL, an H3 analogue, consisting of 12 residues (N-terminal)

As shown in figure 2 , all three peptides do bind to LC1.

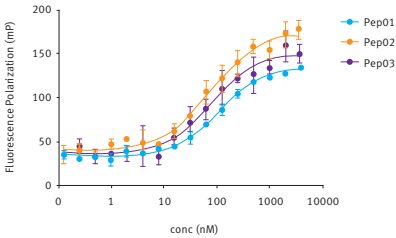

Fig. 2: **Direct binding assay for LC1 and H3-derived peptides.** In blue (pep01) H3 tail, 21 aa, methyl-Lys. In orange (pep02) H3 tail, 21 aa, K4M. In purple (pep03) SNAIL short peptide, 12 aa. Different amplitudes in the curves might be due to different protein-ligand conformations in solution.

Acquired data was analysed using Mars Software and graphically edited in GraphPad. Association constants were calculated modifying the equation for fluorescent anisotropy (see equation 1) using constant label concentration as constrain.

$$B = \frac{L_T + K_d + R_T - \sqrt{[(L_T + K_d + R_T)^2 - 4L_T R_T]}}{2}$$

Eq. 1: **Fitting for Fluorescence Anisotropy.** B= binding, Lt = total ligand concentration, Rt= total "receptor" concentration.

To further assay the binding of histone tail derivate peptides and to confirm association parameters, we proceeded using a competition approach: for this set of experiments a new test mix was prepared, as described above. We titrated in different potential competitors, using the same enzyme concentration, which depended on Kd values obtained from direct binding assays. As shown below (Fig. 3), we were able to assess affinity of different potential binders using a different approach but still in fluorescence polarization.

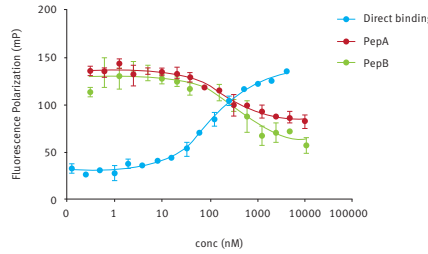

Fig. 3: Competitive Assays for peptide binding to LC1 complex. In blue, the reference direct binding curve is shown. In green and red are potential binders to the active site of LC1 that show a similar affinity for the enzyme.

Table 1 enlists all molecules used in both direct and competitive assays with the resulting affinities values (Kd).

Table 1: Summary of association constants for LC1-peptides binding. For pep01-02-03, the different constants are to be considered coherent, as differences might be due to the sensitivity of the measurement and in the mix preparation.

Ligand	Kd	Type of Assay
Pep01	107.3 nM ± 7.6 nM	Direct Binding
Pep02	76.4 nM ± 10.4 nM	Direct Binding
Pep03	81.9 nM ± 16.7 nM	Direct Binding
Pep A	147.1 nM ± 35.4 nM	Competition
Pep B	316.6 nM ± 59.9 nM	Competition

Conclusion

Fluorescence Polarization has proven to be a reliable technique to probe protein-ligand interaction in real-time and in solution. Both, direct and competitive assays, are useful tools to analyse binding properties. Next to the CLARIOstar very similar results were also obtained on the PHERAstar® *FS* microplate reader.

Transcreener® ADP2 FP assay certification for BMG LABTECH instrumentation

Meera Kumar[1], Franka Maurer[2] and E.J. Dell[2]
[1]BellBrook Labs; [2]BMG LABTECH

- **Transcreener® ADP2 Assay kit is a far-red competitive FP immunoassay based on the detection of ADP**
- **Transcreener® can monitor any enzymatic reaction that produces ADP (ATP range is 0.1 - 1000 µM)**
- **PHERAstar® *FS*, CLARIOstar® and POLARstar® Omega validated by BellBrook Labs**

Introduction

BellBrook Labs offers a variety of high throughput screenings assays for enzymes. The Transcreener® ADP² FP assay can be used to detect the activity of any kinase or ATPase. This simple ADP detecting method is universal for all ADP-producing enzymes and can be used with any substrate.
In this application note we show ADP/ATP standard curves created during performing the Transcreener® assay using different microplate readers from BMG LABTECH.

Assay Principle

The Transcreener® ADP² Assay is a fluorescence polarization immunoassay based on the detection of ADP by an antibody (Figure 1). This assay platform provides the possibility to universally interrogate all enzymes that catalyze group transfer reactions with ATP. In step one of the assay, enzymes catalyze the transfer of phosphate from ATP to a protein, peptide, lipid or small molecule resulting in the accumulation of ADP.

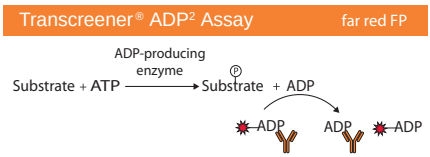

Fig. 1: Transcreener ADP² Assay Principle for Kinases.

In step two the Transcreener® ADP² Detection Mixture, which contains an ADP Alexa633 tracer bound to an anti-ADP antibody, is added. If there is enzymatic activity resulting in necessary ADP then the bound tracer is displaced by the ADP. The free tracer rotates quickly leading to a lower polarization value. If there is no free ADP because of no enzymatic activity, the tracer is still bound to the antibody. This whole construct rotates very slowly giving a higher polarization number. Therefore, ADP production leads to a decrease in fluorescence polarization.

Materials & Methods

- Black 384 well and 1536 well microplates from Corning
- Black 96 well half area flat bottom polystyrene NBS™ microplate, Corning
- Transcreener® ADP² Assay from BellBrook Labs, Madison, WI, (including ADP Alexa633 Tracer, ADP² Antibody, Stop & Detect Buffer B, ATP, and ADP)

Standards preparation

Transcreener® HTS assay performance were identified by running a 10 µM ATP/ADP and 0.1 µM ATP/ADP standard curve (24 replicates), as standard curves of this type mimic enzyme reactions. Starting with 10 µM or 100 nM ATP, ADP was added in increasing amounts and ATP is decreased proportionately, maintaining a total adenine nucleotide concentration of 10 µM and 100 nM respectively.

ADP Detection Mixture

This solution contains 4 nM tracer, 1x stop and detect buffer, and 15 µg/ml (for 10 µM) and 1 µg/ml (for 100 nM). The ADP detection mixture is diluted two fold in the well which leads to the following final concentrations in the well: 2 nM tracer, 0.5x buffer and antibody.

Antibody concentration

The final antibody concentration per well was 0.5 µg/ml (100 nM standard curve) and 7.5 µg/ml (10 µM standard curve). Please note that the optimal antibody concentration can differ significantly depending on the enzymatic reaction conditions. For optimal assay performance it is necessary to do an antibody titration under the specific enzyme and buffer conditions used in your experiment.

Instrument settings

10 µl of standard and 10 µl of detection mixture were mixed in a 384-well microplate which was sealed and incubated at room temperature for 1 hour. After incubation the sealer was removed and the plate was measured. For the 96-well half area plate the final volume in the well was 140 µl. For the 1536-well plate the final volume in the well was 8 µl.

	FLUOstar/ POLARstar Omega	CLARIOstar	PHERAstar *FS*
Detection mode	Fluorescence Polarization		
Method	Endpoint, Top optic		
Well Format	96-well	96-well 384-well 1536-well	96-well 384-well 1536-well
Optic settings	Ex-Filter: 630-10 Em-Filter: 670-10	Ex-Filter: 590-50 Em-Filter: 675-50	Transcreener specific FP optic module: FP 590 675 675
mP target value	Was set to be 20 mP for a well containing the free tracer		

Rev. 12/2014
Keywords: **ADP, Alexa633, ATP, Competitive immunoassay, Transcreener**

Results & Discussion

Figure 2 shows the ADP/ATP standard curve measured in the 96 well format. Graphing on the log scale eliminates the point that corresponds to zero. To include all twelve points along the curve, the value for 0 µM ADP/10 µM ATP was graphed at 0.01 µM position.

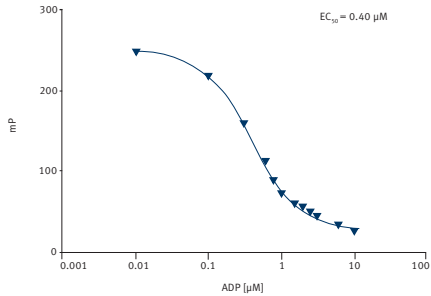

Fig. 2: ATP/ADP standard curve performed in a 96-well half area microplate. Data was measured on a POLARstar Omega.

Figure 3 and 4 show the standard curves measured in 384 well and 1536 well format, respectively.

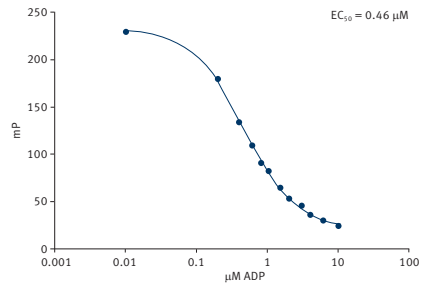

Fig. 3: ATP/ADP standard curve in a 384 well microplate. Data was measured on a PHERAstar *FS*.

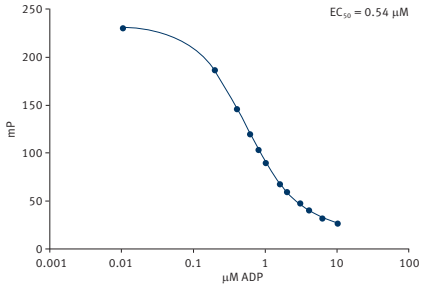

Fig. 4: ATP/ADP standard curve in a 1536 well microplate. Data was measured on a PHERAstar *FS*.

Conclusion

The universally generic nature of the Transcreener® ADP² kit will reduce assay development efforts thus allowing HTS to occur earlier. As a characteristic parameter for the quality of the assay, a Z′ value > 0.7 was calculated, which represents an excellent assay performance. Z′ values between 0.5 and 1 indicate a highly robust screening assay and reflect high quality of instrumentation.

Based on the data exemplarily shown in this application note, the POLARstar Omega, the CLARIOstar as well as the PHERAstar *FS* were certified for the Transcreener® ADP² FP assay.

Transcreener® is a patented technology of BellBrook Labs.

Fluorescence polarization based assay suitable for screening for H-Prostaglandin D Synthase inhibitors

Carl Peters
[1]BMG LABTECH, Cary, NC

- **The Prostaglandin D Synthase (hematopoietic-type) FP-Based Inhibitor Screening Assay Kit provides a robust and easy to use assay**
- **Both the CLARIOstar® and PHERAstar® *FS* excel at detection of this fluorescence polarization based assay**

Introduction

Prostaglandins are a group of lipid compounds that are involved in diverse effects in animal physiology. Prostaglandin D2 (PGD2) has been characterized for its role in asthma where its concentration has been shown to be 10 times higher in asthma patients leading to bronchial airway contraction after exposure to an allergen. For this and other reasons inhibitors are sought for the hematopoietic prostaglandin D synthase (H-PGDS) which catalyzes the final PGD2 biosynthesis step.
Here we show the performance of Cayman Chemicals green FP-based inhibitor assay screening kit. The kit was tested with both the CLARIOstar and PHERAstar *FS*. Settings more suitable for high throughput screening (HTS) were also tested.

Assay Principle

The Prostaglandin D Synthase FP-Based Inhibitor Screening Assay provides a convenient one step assay. An H-PGDS inhibitor was conjugated to fluorescein and other inhibitors are discovered by their ability to displace this probe from binding with H-PGDS (Figure 1).

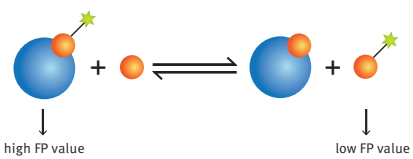

Fig. 1: H-PGDS Green FP Inhibitor Assay Principle.

When a fluorescein conjugated probe interacts with H-PGDS a high FP signal is detected. Inhibitors disrupt binding of the probe and the free probe exhibits a low FP signal.

In the absence of an effective inhibitor the probe will remain bound to H-PGDS thus reducing the rotational freedom of the probe, resulting in a high FP signal. Increasing concentrations of an effective inhibitor will lead to an increased amount of displaced probe. The displaced probe has an increased rotational freedom and therefore a lower FP signal.

This assay prevents the need for the traditional multistep assay that utilizes the highly unstable PGD2-precursor PGH2.

Materials & Methods

- Prostaglandin D Synthase (hematopoietic-type) FP-Based Inhibitor Screening Assay Kit – Green (Cayman Chemicals # 600007)
- PHERAstar *FS* and CLARIOstar microplate readers (BMG LABTECH)
- Inhibitors and additional reagents were purchased from commercial sources

To test kit function 16 point titration curves were prepared for 2 known H-PGDS inhibitors; HQL-79 (provided in the kit) and TFC 007. In addition a similar titration curve was prepared for AT 56 an inhibitor of lipocalin-type PGDS (L-PGDS). For all 2-fold serial dilutions were made in DMSO.

Kit reagents were prepared according to kit instructions to create an assay cocktail. 47.5 µl of this cocktail was pipetted into the wells of a 384-well plate. This was followed by 2.5 µl of the appropriate inhibitor concentration or DMSO as a negative inhibition control. The final concentration of the 1st point in the titration curve was 2.5 mM for AT 56, 250 µM for HQL-79 and 25 µM for TFC 007. Following an incubation of 90 minutes at room temperature the plates were read using the following settings:

CLARIOstar settings:

Measurement method:	Fluorescence Polarization, End Point
Filter Settings:	482-16 / LP 504 / 530-40
Settling Time:	0.1
Number of flashes:	200
Focus and Gain:	Adjusted prior to measurement
Target mP:	200 mP set on DMSO control

PHERAstar FS settings:

Measurement method:	Fluorescence Polarization, End Point
Optic Module:	FP 485 520 520
Settling Time:	0.3 or 0.1
Number of flashes:	200 or 10
Focus and Gain:	Adjusted prior to measurement
Target mP:	200 mP set on DMSO control

Results & Discussion

We first wanted to confirm that the appropriate filters were selected by performing a spectral scan of the H-PGDS FP Fluorescent Probe – Green (Figure 2). The fluorescein conjugated probe behaves as expected, confirming the selection of CLARIOstar filters and of PHERAstar *FS* optic module.

Rev. 07/2015
Keywords: **Asthma, Inhibitor dose response, Prostaglandins, Protein-protein interaction, Spectral scan**

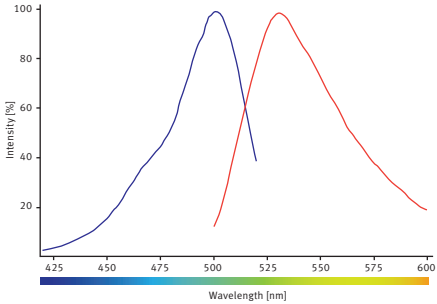

Fig. 2: H-PGDS FP Probe - Green Spectral Scan. Probe was scanned using a Fluorescence spectrum measurement. Excitation scan from 420-10 to 520-10 using 548-16 as the emission wavelength. Emission scan from 500-10 to 600-10 using 472-16 as the excitation wavelength.

The response to inhibitor treatments detected with the CLARIOstar is shown in Figure 3. The assay exhibits high quality based on the calculated Z' value of 0.84.

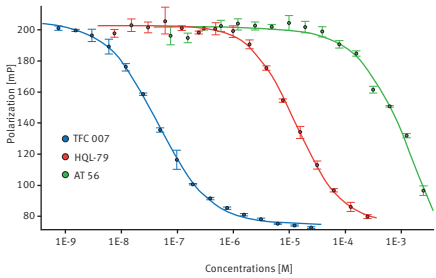

Fig. 3: Inhibitor titration curves on CLARIOstar. 4-parameter fit curves for TFC 007 (R^2=0.999), HQL-79 (R^2=0.998) and AT 56 (R^2=0.984).

From the 4-parameter fit curves we can determine the IC_{50} for inhibition of H-PGDS. TFC 007 exhibits an IC_{50} of 45 nM which corresponds well with the reported value of 83 nM[1]. HQL-79 is reported to have an IC_{50} of 6 μM[2] and in this experiment exhibited an IC_{50} of 13 μM. AT 56 is an L-PDGS selective inhibitor and had previously been reported to have no effect on H-PGDS at concentrations as high as 100 μM[3]. The results shown here predict an IC_{50} of 1.9 mM but would require further analysis at higher concentrations.

The PHERAstar *FS* provides similarly robust detection (Table 1). We therefore tested whether settings more conducive to HTS could be employed. Figure 4 shows results using 10 flashes which indicate that the PHERAstar *FS* maintains performance predicting similar IC_{50} values to those seen with the CLARIOstar.

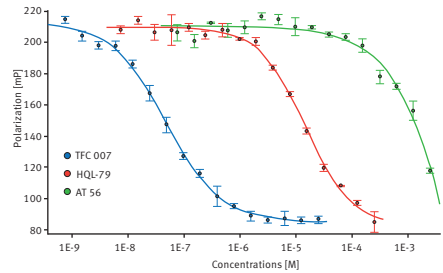

Fig. 4: Inhibitor titration curves on PHERAstar *FS*. 4-parameter fit curves for TFC 007 (R^2=0.998, IC_{50}=48 nM), HQL-79 (R^2=0.997, IC_{50}=15 μM) and AT 56 (R^2=0.969, IC_{50}=1.25 mM).

Table 1 shows that outstanding assay quality is maintained in the PHERAstar *FS* even though HTS settings are used.

Flashes / Settling Time	Z'	384-well read time
200/0.3	0.82	7 minutes
10/0.1	0.74	1 minute 33 sec

Conclusion

Both the CLARIOstar and PHERAstar *FS* exhibit excellent assay quality when used to read Cayman's Prostaglandin D Synthase FP-Based Inhibitor Screening Assay. Furthermore the PHERAstar *FS* maintains this assay quality even when using settings more suitable for high throughput.

References

1. Nabe T., et al. (2011) *Prostaglandins Other Lipid Mediat.* **95**(1-4) 27-34
2. Artiake K. et al. (2006) *J. Biol. Chem.* **281(22)** 15277-15286.
3. Irikura, D., et al. (2009) *J. Biol. Chem.* **284(12)** 7623-7630.

PHERAstar® *FSX* certified for Transcreener® assays

Franka Maurer[1] and Meera Kumar[2]
[1]BMG LABTECH GmbH, Ortenberg, Germany [2]BellBrook Labs, USA

- **Detect enzyme activity in HTS format**
- **PHERAstar® *FSX* equipped with Transcreener specific optic modules to measure signals of far-red dyes**
- **High sensitivity data with read times < 30 seconds for a whole 384-well microplate**

Introduction

The Transcreener® assays from BellBrook Labs offer a flexible approach to detect enzyme activity in high throughput screening (HTS) format. The assay is based on the direct detection of nucleotides such as ADP, GDP, UDP, GMP and AMP allowing determining the activity of e.g. methyltransferases, acetyltransferases, kinases or GTPases. The assays use far-red dyes and are available in three detection modes:

- Transcreener FP (Fluorescence Polarization)
- Transcreener FI (Fluorescence Intensity)
- Transcreener TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer)

In this application note we show the performance of the PHERAstar *FSX* microplate reader from BMG LABTECH in Transcreener® assays. In validation measurements the output values resulted in high sensitivity data while the read time per 384-well microplate can be < 30 sec. confirming the instrument to be an excellent option for HTS.

Assay Principle

After the enzymatic reaction ADP is present in the sample. A general description of the different kinds of ADP detection is presented in Fig. 1.

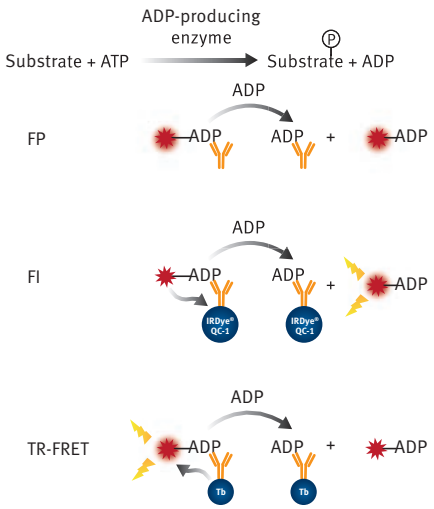

Fig. 1: Assay principle.

Transcreener FP principle
ADP-labeled with Alexa633 and conjugated to an antibody is added to the well. ADP that is produced during the enzymatic reaction will compete with the Alexa633-ADP for the binding site of the ADP antibody and will displace the Alexa633-ADP. This will result in a decreased FP value.

Transcreener FI principle
ADP-labeled with Alexa594 is conjugated to an antibody that carries a quencher. ADP that is produced during the enzymatic reaction will compete with the Alexa594-ADP for the binding site of the ADP antibody and will displace the Alexa594-ADP. That brings fluorescent dye and quencher into distance resulting in an increase in FI value.

Transcreener TR-FRET principle
ADP-labeled with acceptor dye HiLyte647 is conjugated to an antibody that carries donor terbium chelate. ADP that is produced during the enzymatic reaction will compete with the HiLyte647-ADP for the binding site of the ADP antibody and will displace the labeled ADP. That brings TR-FRET donor and acceptor out of proximity and leads to a decrease in TR-FRET signal.

Materials & Methods

- Transcreener® ADP² FP, FI, TR-FRET assay kits
- Black 384-well small volume, low binding plate (Greiner)
- PHERAstar *FSX* microplate reader

ADP/ATP standards were prepared to mimic an enzymatic reaction. 10 µM ADP and 10 µM ATP stock solutions were combined at varied proportions to create different percent conversions of ATP to ADP ranging from 0 to 10 µM. 10 µl of standard and 10 µl of ADP detection mixture (containing ADP antibody and ADP far red tracers) are combined in the microplate and incubated for 1 hour at room temperature. After incubation the plate was measured in the PHERAstar *FSX*.

PHERAstar FSX instrument settings

	Transcreener FP	Transcreener FI	Transcreener TR-FRET
Simultaneous Dual Emission	yes		yes
Optic module	FP 590 675 675	FI 580 620	TRF 337 665 620
Excitation source	Flash lamp	Flash lamp	Laser or flash lamp
			Int. start: 50 µs Int. time: 50 µs
Gain/Focus	Should be adjusted prior the measurement		

Rev. 02/2016
Keywords: **ADP, ATP, BellBrook Labs, Kinase, Transcreener**

Results & Discussion

Transcreener FP

Fig. 2 shows that with the PHERAstar *FSX* an assay window higher than 200 mP has been reached (assay window = values for 100 % ATP- values for 100 % ADP).

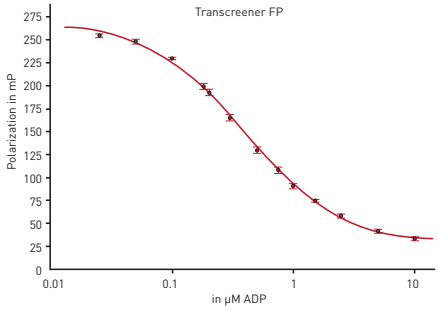

Fig. 2: 10 µM ADP standard curve of the Transcreener FP assay.

The effect of the number of flashes on robustness of measurement is shown in Table 1.

No. of flashes	100	50	25	13
Δ mP at 10 % conversion	156	157	158	158
Z' at 10 % conversion	0.878	0.819	0.780	0.749
Read time for a full 384w plate	3 min 31 sec	2 min 26 sec	1 min 53 sec	1 min 37 sec

The assay can be reliably measured in less than 2 min for a whole 384-well microplate.

Transcreener FI

Transcreener FI signals increase with increasing ADP concentrations. According to the certification requirements it is necessary to obtain a Z´ factor of 0.7 at 10 % ATP conversion. Fig. 3 shows that with the PHERAstar *FSX* this Z´factor is achieved at 4% conversion, much lower than the certification requirements.

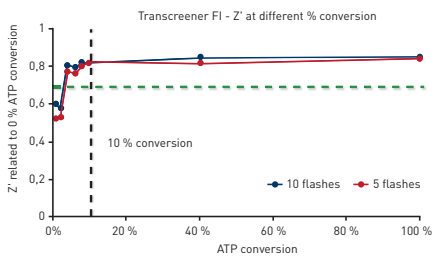

Fig. 3: Z´values obtained in a standard curve mimic conversion of 10 µM ATP to ADP.

Transcreener TR-FRET

TR-FRET measurements can be performed by either using the flash lamp or the laser in the PHERAstar *FSX*. A standard curve obtained with the laser is shown in Fig. 4.

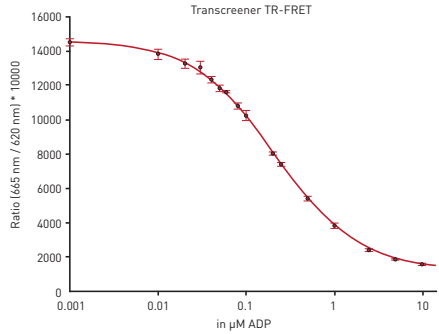

Fig. 4: 10 µM ADP standard curve of the Transcreener TR-FRET assay.

Flash lamp and laser give comparable data in terms of assay window and stability of data. The advantage of the laser is the use of a less number of flashes. In the flying mode the whole plate can be read in less than 30 sec (Table 2).

Flashes Flash lamp	Z' value	Read time	*Flahes Laser*	Z' value	Read time
50	0.815	2 min 27 sec	10	0.896	2 min 21 sec
25	0.807	1 min 53 sec	5	0.884	1 min 49 sec
10	0.747	1 min 33 sec	1	0.830	1 min 23 sec
5	0.746	1 min 26 sec	flying	0.736	27 sec

Validation criteria from BellBrook Labs

- 384-well format
- Z´- Factor ≥ 0.7 at 10 % conversion of 10 µM ATP
- Δ mP ≥ 95 mP at 10 % conversion of 10 µM ATP
- Read Times to achieve specifications ≤ 5 minutes

Conclusion

Based on the data shown the PHERAstar *FSX* has obtained all three Transcreener certifications.

PHERAstar® *FSX*
PHERAstar® *FS*

Homogeneous IgG AlphaLISA® assay performed on BMG LABTECH's PHERAstar® *FS*

Franka Ganske[1] and Keith Ansell[2]
[1]BMG LABTECH, Offenburg, Germany; [2]MRC-T, Burtonhole Lane, London, UK

- **IgG AlphaLISA® assay performed on BMG LABTECH's PHERAstar® *FS***
- **Z'value of 0.92 indicates a highly robust assay combined with high quality instrumentation**
- **IgG limit of detection determined to be < 0.21 ng/mL**

Introduction

Immunoglobulins or antibodies are proteins that are involved in the immune response. They bind with high affinity at exogenous substances (antigens). Immunoglobulins (Ig) are classified according to the structure. The most present antibody in plasma is IgG. It is part of the secondary antibody response and is built 3 weeks after antigen recognition. There are several assays for the determination of IgG commercially available. This application note shows the detection of IgG using an AlphaLISA® assay performed on BMG LABTECH's PHERAstar *FS*.

Assay Principle

The AlphaLISA® technology from PerkinElmer is based on the AlphaScreen® detection method: upon laser excitation at 680 nm of Donor beads ambient oxygen is converted into singlet oxygen released at a rate of up to 60,000 molecules per second. Singlet oxygen molecules have a short lifetime (4 µs in aqueous solutions) and diffuse of no more than 200 nm. When a biomolecular interaction brings the Donor and Acceptor beads in proximity, the singlet oxygen reaches the Acceptor bead and a cascade of chemical reactions is initiated producing a greatly amplified luminescence signal. The assay principle of the IgG AlphaLISA® assay is given in Figure 1.

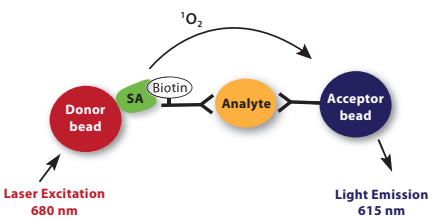

Fig. 1: Principle of the IgG (Analyte) AlphaLISA® assay kit.

Anti-IgG antibody coated Acceptor beads and biotinylated anti-IgG antibody bind to IgG. In a second step streptavidin-coated Donor beads are added and bind to the biotylated anti-IgG antibody. Donor and Acceptor beads are in close proximity when IgG is present in the sample. After laser excitation at 680 nm a cascade of chemical reactions is started resulting in a luminescence signal at 615 nm. If no IgG is present, no light emission is detected.

Materials & Methods

- AlphaLISA® IgG Kit, PerkinElmer
- White opaque 384-well OptiPlate™, PerkinElmer
- White 384-well small volume plate, Greiner
- PHERAstar *FS* microplate reader, BMG LABTECH

The IgG AlphaLISA® standard curve was performed in accordance with the kit protocol. Briefly, a serial dilution of lyophilized IgG was prepared in AlphaLISA® immunoassay buffer. A mix of Acceptor beads and biotinylated antibody were added to each of the 12 standards and to the blank (contains no IgG). Following 60 min incubation at room temperature, Donor beads were added. As Donor beads are light sensitive this step has to be done under subdued or green light. After another 30 min of incubation in the dark the AlphaLISA® signal is measured in the PHERAstar *FS*. BMG LABTECH has developed an AlphaLISA® specific optic module and the instrument settings for a 384-well plate can be found below.

Instrument Settings	
Measurement method	AlphaScreen®
Reading mode	Endpoint
Optic module	AlphaLISA® 680 615

General Settings	
Positioning delay	0.10 s
Excitation time	0.30 s
Integration start	0.34 s
Integration time	0.30 s
Gain	3600

850 µL of each standard and blank were prepared in vials. After incubation a certain amount of reaction mix was transferred into the two different types of 384-well microplates. 50 µL were used for the 384-well standard plate and 17 µL were the final volume in the 384-well small volume plate. Each plate consisted of 12 replicates for both the standards containing different IgG concentrations and the blank. Because of the different fill heights it is recommended to perform a focus adjustment once for every plate type. The focal height will be optimized resulting in the highest possible sensitivity.

Results & Discussion

Figure 2 shows an IgG standard curve obtained in 384-well plates using a volume of 50 µL.

Rev. 03/2009
Keywords: **Antibody, IgG, IgM, Immune response, Immunoglobulins**

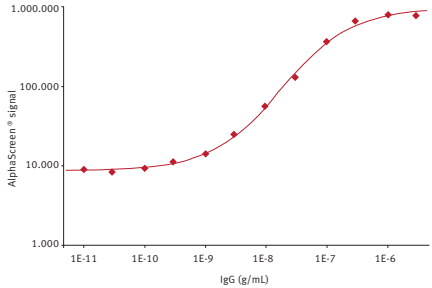

Fig. 2: A typical log-log IgG standard curve recorded on the PHERAstar *FS* using the AlphaLISA® specific optic module in 384-well format.

For low concentrations the linear - linear scale is preferred (Figure 3). With the MARS data analysis software both standard curves can easily be plotted.

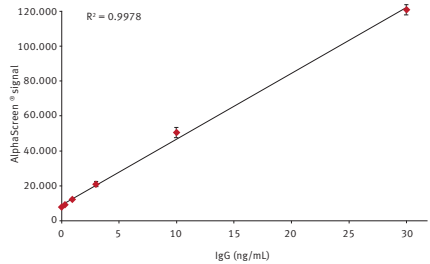

Fig. 3: A linear-linear chart at low IgG concentrations obtained with the MARS data and analysis software.

Z' values, a standard in evaluating HTS methods, and the limit of IgG detection were calculated for both plate types (Table 1). The formulas for both calculations are given below.

$$Z' = 1 - \frac{3\sigma_p + 3\sigma_n}{|\mu_p - \mu_n|}$$

where μ_p = mean of "positive control" (max ratio), μ_n = mean of "negative control" (min ratio), and σ = the corresponding standard deviations.
The limit of detection (LOD) was determined by interpolating the average blank values + 3x the standard deviation of the blank on the standard curve (4-parameter fit).

Plate Format	384-well standard plate 50 µL	384-well small volume plate 17 µL
Z'value	0.92	0.91
Limit of IgG detection (ng/mL)	0.21	0.27

Table 1: Z' values and LOD of IgG in 50 µL and in 17 µL.

Conclusion

The IgG AlphaLISA® detection kit was successfully performed on the PHERAstar *FS*. The sensitivity stated by the kit manufacturer was reached in 384-well standard plates and was slightly better than in 384-well small volume format. Nonetheless, we could show that it is possible to decrease the assay volume to 17 µL with good sensitivity and very good Z' values. The multidetection HTS reader PHERAstar *FS* shows great performance in AlphaScreen® and AlphaLISA® mode in 384-well small volume plate format. The easy to use software alows simple assay optimization regarding sensitivity and read times. The PHERAstar *FS* has been designed to read all HTS detection modes (fluorescence intensity, time-resolved fluorescence, fluorescence polarization, luminescence, AlphaScreen®, and absorbance) in all plate formats up to 1536 wells.

PHERAstar® *FSX*
PHERAstar® *FS*

CLARIOstar®

Omega Series

An AlphaScreen® SureFire® Phospho-ERK1/2 assay

J.D. Brown, R. Mould and C.J. Langmead
Department of Pharmacology, Heptares Therapeutics

- **ERK phosphorylation cascade can be utilized to measure Gi and Gq GPCR**
- **The AlphaScreen® SureFire® Phospho-ERK1/2 assay was performed in 384-well format**
- **Dose response curves for Gi and Gq agonists are shown**

Introduction

G-protein coupled receptors (GPCRs) are a large family of receptors which have a broad involvement in cellular responses affecting many important body functions both in health and disease. On activation GPCRs signal through different G-proteins (e.g. Gs, Gi or Gq) to elicit a cellular response which can be mediated through the activation of a number of different signaling cascades which include intracellular Ca^{2+} mobilization and the formation or inhibition of adenylate cyclase: Although these technologies have been successful in many cases they have had their limitation. Responses for many GPCRs such as Gi coupled GPCRs are not easily measured in current assay formats and other methods have been investigated as alternative readout. One such pathway is the ERK phosphorylation cascade, which results in the phosphorylation of both cytoplasmic and nuclear proteins regulating gene transcription. Hence this type of assay has the ability to measure cellular changes activated by Gs, Gi anf Gq coupled receptors giving potential to use a single assay format for screening.

Here a microplate reader equipped with a flash lamp is used to detect ERK phosphorylation using a AlphaScreen® SureFire® proximity-based assay. ERK phosphorylation is demonstrated at receptors which signal via Gi and Gq G-proteins.

Assay Principle

The AlphaScreen® assay uses the diffusion of singlet state oxygen from Donor to Acceptor beads. Upon excitation at 680 nm of Donor beads ambient oxygen is converted into singlet oxygen released at a rate of up to 60,000 molecules per second. Singlet oxygen molecules have a short lifetime (4 µs in aqueous solutions) and diffuse of no more than 200 nm. When a biomolecular interaction brings the Donor and Acceptor beads in proximity, the singlet oxygen reaches the Acceptor bead and a cascade of chemical reactions is initiated producing a greatly amplified luminescence signal in the range of 520 – 620 nm. The AlphaScreen® SureFire® ERK1/2 phosphorylation assay is based on an immuno-sandwich assay principle (Figure 1).
A phosphorylated cellular protein (analyte) is sandwiched between a streptavidin (SA)-coated Donor bead associated with an analyte antibody and a Protein A-conjugated Acceptor bead associated with an anti-phospho specific antibody. Phosphorylation of the analyte results in an increase in the luminescence signal.

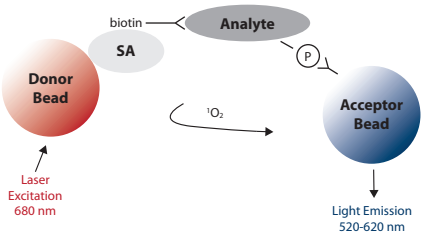

Fig. 1: Assay Principle of the AlphaScreen® SureFire® Phospho-ERK1/2 assay.

Materials & Methods

- Microplate reader from BMG LABTECH equipped with the AlphaScreen® option
- AlphaScreen® SureFire® ERK1/2 phosphorylation assay kit, PerkinElmer
- AlphaScreen® Protein A general IgG detection kit, PerkinElmer
- Proxiplate™ -384 plus, white, 384-shallow well plate, PerkinElmer
- 96-well half area tissue treated cell culture plate, Corning

CHO cells stably expressing either a Gq or Gi coupled GPCR were plated at a cell density of 25K per well in 96 well half area tissue culture treated plates and cells were allowed to attach for 4h at 37°C. After 4h media was removed and replaced with 45 µL of DMEM/F12 w/o FBS to allow quiescence of the cells. Cells were incubated for 18h and then the assay performed. 5 µL of test compound was added and incubation time varied for time course experiments while kept constant, 5 min, for concentration response curve experiments. Following the incubation period, culture medium was removed and cells lysed with lysis buffer. Lysates were then transferred to a white 384-shallow well Proxiplate™ and AlphaScreen® beads were added in a single pipetting step in accordance with the kit protocol. The plate was incubated for 2h and read on the microplate reader. Data were expressed as % maximal response obtained with 0.3% FBS.

Instrument Settings

Measurement method:	AlphaScreen
Reading Mode:	Endpoint
Filters:	Ex AS/Em AS

General Settings

Excitation time:	0.30 s
Integration start:	0.34 s
Integration time:	0.60 s

Rev. 01/2011
Keywords: **ERK1/2, GPCR, Phosphorylation, Receptor, SureFire**

Results & Discussion

Initial experiments were designed to look at the optimal time of stimulation for both receptors.

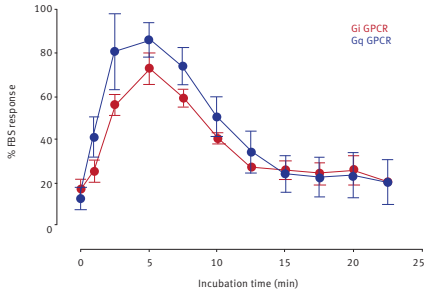

Fig. 2: FBS response dependent on incubation time.

Agonist stimulation gave a transient increase in pERK1/2, peaking at 5 min for both Gi and Gq receptor activation. Using the 5 min stimulation time point standard agonists were profiled in the concentration response curve format (Fig. 3A und B).

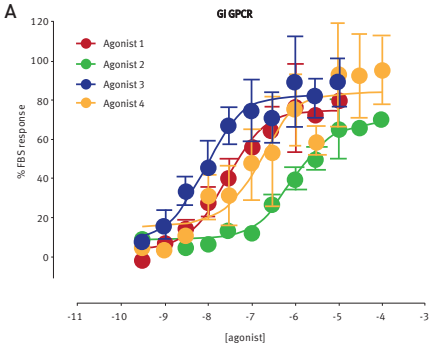

Fig. 3A: AlphaScreen® measurement of activated Gi coupled GPCRs.

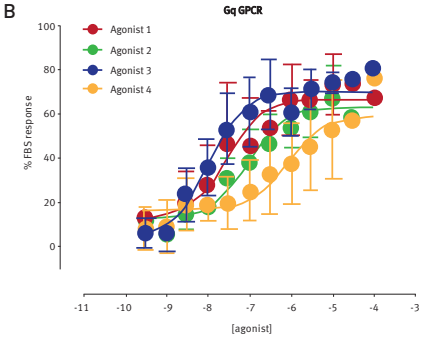

Fig. 3B: AlphaScreen® measurement of activated Gq coupled GPCRs.

The AlphaScreen® SureFire® ERK1/2 phosphorylation assay system is able to detect G-Protein coupled receptor signaling and can be robustly measured using the BMG LABTECH microplate reader.

Conclusion

The AlphaScreen® SureFire® ERK1/2 phosphorylation assay was successfully performed in 384-well format.

PHERAstar® *FSX*
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Omega Series

Miniaturization of a cell-based TNF-α AlphaLISA® assay using Echo® liquid handler and the PHERAstar® *FS*

Carl Peters[1] and Bonnie Edwards[2]
[1]BMG LABTECH, Cary, NC, USA; [2]Labcyte, Sunnyvale, CA, USA

- **AlphaLISA assay can be miniaturized down to 2 µl in 1536-well microplates**
- **TNF-α was detected in stimulated THP-1 cells and antagonist potency was determined**

Introduction

Tumor necrosis factor (TNF)-α is mainly secreted by macrophages and monocytes where it plays an important role in several functions of the immune system, such as inflammation and cell death. However, overproduction of TNF-α has been linked to inflammatory disorders such as rheumatoid arthritis.Therefore, screening for potential inhibitors of TNF-α production could lead to beneficial treatments for inflammatory diseases.

Miniaturization for screening purposes presents an attractive scenario in which drug discovery can take place while saving on expenditure of target compounds and detection reagents. For assay miniaturization to be successful liquids must be handled with extreme accuracy and the small volumes require highly sensitive detection. Here we describe the use of the PHERAstar® *FS* from BMG LABTECH to detect an AlphaLISA® assay which has been miniaturized by the Echo® Liquid Handler from Labcyte.

Assay Principle

The TNF-α AlphaLISA® Immunoassay uses antibodies specific for the analyte (TNF-α) that are coupled to donor and acceptor beads. If the analyte is present both donor and acceptor beads will bind via their antibodies to the analyte. That brings donor and acceptor beads into proximity such that an emission signal at 615 nm can be detected following laser excitation at 680 nm.

The Echo® Liquid Handler from Labcyte employs sound energy to transfer liquids in nanoliter volume increments with precision and accuracy. The transfer is tipless, eliminating the risk of carryover while offering additional cost savings.

Laser excitation and assay specific optic modules available in the PHERAstar *FS* microplate reader enhance performance of AlphaLISA assays.

Materials & Methods

- AlphaLISA® Human TNF-α Research Immunoassay Kit (PerkinElmer)
- THP-1 cells (ATCC)
- (-)-Isoproterenol hydrochloride, salbutamol, histamine dihydrochloride and lipopolysaccharides (LPS) were all obtained from Sigma
- Echo 555 Liquid Handler and 384-well Echo qualified source microplates (Labcyte)
- 1536-well white, solid-bottom assay microplate (Labcyte)
- PHERAstar *FS* microplate reader (BMG LABTECH)

For all experiments liquid was added using the Echo® Liquid Handler unless otherwise noted. After every transfer step a brief centrifugation was done (1 sec/ 1000 rpm) before the plate was sealed or lidded.

TNFα standard dilution series
Initial miniaturization experiments were performed to ensure the ability to detect known amounts of analyte. To accomplish this 1.6 µl of analyte was added followed by 200 nl of a mixture containing acceptor beads and anti-TNF-α antibody. Following a one hour incubation at room temperature in the dark 200 nl of donor beads was added for a final volume of 2 µl. After an additional 30 minute room temperature incubation in the dark plates were read on the PHERAstar *FS*.

TNFα production in THP-1 cells
Subsequent experiments sought to assess the ability to detect TNF-a production by THP-1 cells and the effect of various compounds on the production of TNF-α. First 5 nl of compound was transferred to plates. Then 3000 cells were dispensed in 1.5 µl using a Multidrop™ Combi followed by addition of 95 nl of LPS. Following a three hour incubation at 37°C 200 nl of acceptor beads/ anti-TNF-α antibody was added. After a one hour incubation at room temperature 200 nl of donor beads was added for a final volume of 2 µl. After an additional 30 minute room temperature incubation plates were read on the PHERAstar *FS*.

For both experiments plates were read using the following settings:

Measurement Type:	AlphaScreen®
Reading Mode:	Endpoint
Optic Module:	AlphaLISA®
Excitation wavelength:	680 nm
Emission wavelength:	615 nm
Positioning delay (sec):	0.1
Excitation time (sec):	0.30
Integration start (sec):	0.34
Integration time (sec):	0.60
Gain:	3600

Rev. 12/2014
Keywords: **1536-well, AlphaLISA, AlphaScreen, Miniaturization, TNFα**

Results & Discussion

Several dilutions of TNF-α were tested to confirm the ability of the Echo liquid handler to accurately dispense the AlphaLISA reagents and the PHERAstar *FS* to sensitively detect a 2 µl assay (Figure 1).

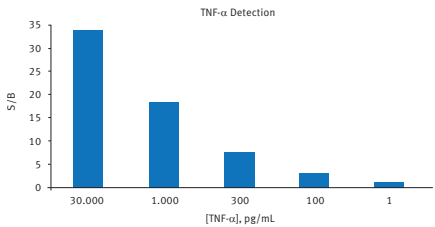


Fig. 1: TNF-α detection in a miniaturized assay.

The results in Fig. 1 indicate that in an assay volume of as little as 2 µl TNF-α was detected across a range of concentrations with high signal to background and sensitivity. Fig. 2 shows the high linearity that is obtained at low analyte concentrations.

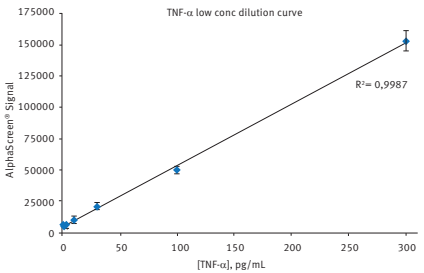


Fig. 2: Linear relationship of low TNF-α concentration and AlphaScreen®/AlphaLISA® signal. Error bars refer to triplicates.

Because of the use of donor and acceptor beads in Alpha technology it was investigated whether over time there was any variability in this AlphaLISA assay. No significant variability was seen after 1 hour indicating that settling of beads in the source plate was not a problem (Data not shown).

THP-1 cells are a human monocytic cell line well characterized for their ability to produce TNF-α in response to treatment with LPS. Previous results have indicated that beta adrenergic receptor and histamine H2 receptor agonists have an inhibitory effect on LPS induction of TNF-α by monocytes.
The results of this miniaturized assay correspond to those predicted (Figure 3).

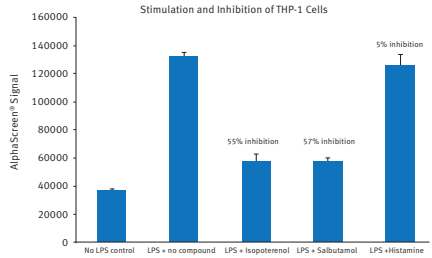


Fig. 3: TNF-α production in THP-1 cells. Cells were stimulated with LPS and inhibition effect is presented for different compounds.

Treatment with LPS exhibits a greater than 3 fold increase in signal while co-treatment with the beta adrenergic receptor agonists leads to greater than 50% inhibition of this increase. Treatment with histamine had a much more modest effect (5% inhibition).

Conclusion

The TNF-α AlphaLISA assay has been successfully miniaturized using Labcyte and BMG LABTECH technologies. The ability to miniaturize these assays and thus reduce reagent use by up to 25 fold, can provide significant cost savings, as well as reduction in compound and sample/cell usage.

PHERAstar® *FSX*
PHERAstar® *FS*

Detection of tyrosine kinase activity in AlphaScreen® mode

Franka Ganske and Marjan Orban
BMG LABTECH

- **AlphaScreen® tyrosine kinase assay performed using either a laser or a flash lamp-equipped microplate reader**
- **high Z' values indicate a highly robust assay combined with high quality instrumentation**
- **Sensitivity determined to be ≤ 100 amol biot-LCK-P per well**

Introduction

Tyrosine kinases are important regulators of cellular processes that include cell cycle progression, metabolism, and apoptosis. Kinases have been found to be involved in e.g. cancer and cardiovascular diseases; therefore, molecules that modulate kinase functions are expected to be promising new drugs. There are different homogeneous technologies which can be used to perform kinase assays. In this application note we will describe the performance of a tyrosine kinase assay using the AlphaScreen® (amplified luminescent proximity homogeneous assay) method.

Assay Principle

The AlphaScreen® assay uses the diffusion of singlet state oxygen from Donor to Acceptor beads. Upon laser excitation at 680 nm of Donor beads ambient oxygen is converted into singlet oxygen released at a rate of up to 60,000 molecules per second. Singlet oxygen molecules have a short lifetime (4 µs in aqueous solutions) and diffuse of no more than 200 nm. When a biomolecular interaction brings the Donor and Acceptor beads in proximity, the singlet oxygen reaches the Acceptor bead and a cascade of chemical reactions is initiated producing a greatly amplified luminescence signal in the range of 520 - 620 nm. The AlphaScreen® P-Tyr-100 assay (figure 1) is based on a sandwich assay principle.

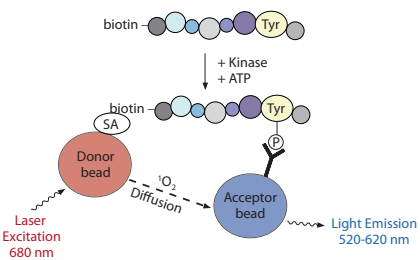


Fig. 1: Principle for an AlphaSreen® tyrosine kinase assay.

After tyrosine kinase phosphorylation, a biotinylated polypeptide substrate is sandwiched between a streptavidin(SA)-coated Donor bead and an anti-phosphotyrosine antibody conjugated Acceptor bead. Phosphorylation of the polypeptide by the tyrosine kinase results in an increase in the luminescence signal.

Materials & Methods

- P-Tyr-100 assay kit, PerkinElmer
- White 384-well small volume plates, Greiner Bio-One

The P-Tyr-100 (Phosphotyrosine) assay kit was performed in AlphaScreen® mode in accordance with the kit protocol in white 384-well small volume plates with a final assay volume of 17 µL. Donor and Acceptor beads were used at a final concentration of 20 µg/mL. The AlphaScreen® components are light sensitive; therefore, the beads should not be exposed to bright light. Beads are best handled under subdued or green filtered light. Plates were read after an hour incubation at room temperature. The instrument settings for a 384-well plate can be found below.

Instrument settings

	FLUOstar®/ POLARstar® Omega	CLARIOstar®	PHERAstar® *FS*
Detection mode	AlphaScreen®		
Method	Endpoint, Top optic		
Optic settings	Ex-Filter: EX AS Em-Filter: Em AS	Ex-Filter: EX AS Em-Filter: Em AS	AlphaScreen Optic module
Excitation time	0.3 seconds		
Integration start	0.34 seconds		
Integration time	0.3 seconds		

Results & Discussion

A nine point titration curve with biotinylated and phosphorylated polypeptide (biot-LCK-P) covering concentrations from 50 nM to 5 pM was performed to demonstrate the kit performance (Figure 2 and Figure 3).

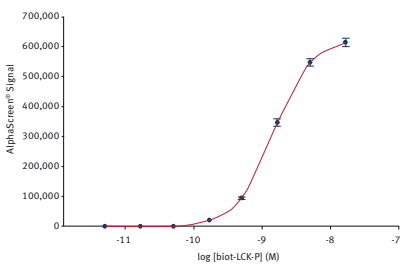


Fig. 2: A typical biot-LCK-P titration curve recorded on the PHERAstar *FS* in AlphaScreen® mode.

Rev. 12/2014
Keywords: **Acceptor beads, AlphaScreen, Beads, Signaling, Tyrosine kinase**

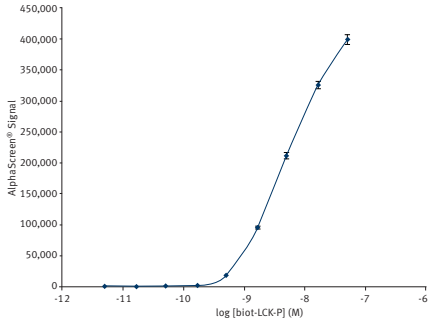


Fig. 3: A typical biot-LCK-P titration curved recorded on the POLARstar Omega in AlphaScreen mode.

The resulting titration curves in Figure 2 and 3 very closely correspond to the curve published in the kit protocol. In order to show that there is no significant well to well variation, the assay was performed with 24 replicates for each concentration and blank. Figure 4 shows the high consistency of well to well measurements.

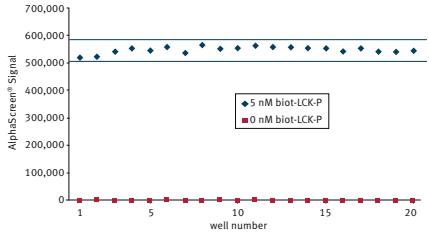


Fig. 4: AlphaScreen® values for 20 replicates at a constant concentration (5 nM) of biotinylated and phosphorylated LCK and a control containing no protein. Data was measured on a PHERAstar *FS*.

Figure 4 shows the high consistency of well to well measurements. The resulting 2.2 %CV (for 5 nM biot-LKC-P) also demonstrates stable measurements. From these assay data, a representative Z' value of 0.93 and an LOD (limit of detection) of ≤100 amol biot-LCK-P per well were calculated.

Conclusion

AlphaScreen® tyrosine kinase assays performed on the PHERAstar *FS* result in very consistent values for replicate wells. As a characteristic parameter for the quality of the assay, a Z' value of 0.93 was calculated, which represents an excellent assay performance. Z' values between 0.5 and 1 indicate a highly robust screening assay and reflect high quality of the instrumentation.

The assay was as well successfully performed on a FLUOstar Omega and a POLARstar Omega in 384-well format. Although there is no laser available, special filters that are optimized for AlphaScreen® provide the possibility to obtain sensitive and consistent data. The Z´-value for the Omega readers were calculated to be > 0.8 while the limit of detection was calculated to be ≤100 amol biot-LCK-P per well.

For the PHERAstar *FS*, the CLARIOstar and the Omega series of readers the limit of detection was identical. This leads to the strong assumption that the assay itself is limiting.

Increasing throughput with dual emission AlphaLISA-AlphaPlex assay and Simultaneous Dual Emission detection

Carl Peters
BMG LABTECH, Cary, NC

- **PHERAstar® *FSX* is an excellent reader for detecting the dual emission AlphaLISA assay**
- **SDE detection enables users to double their productivity**

Introduction

In order to maximize efficiency, screening facilities must constantly strive to increase throughput and minimize sample expenditure, while controlling cost as much as possible. However, saving time, samples and money are irrelevant if in the end the data suffers.

Here we show the performance of the PHERAstar *FSX* in the detection of an AlphaLISA type assay using 2 emission fluorophores. The ability of the PHERAstar *FSX* to perform simultaneous dual emission detection of this assay will allow users to double the information obtained from each well in the same amount of time it takes to run a standard AlphaLISA plate.

Assay Principle

The AlphaLISA principle is quite well characterized and the performance of the PHERAstar series has been described previously in BMG LABTECH Application Note AN260. Recently a modified AlphaLISA has become available that is distinguished by the wavelength of emission with a peak 545 nm as opposed to the traditional 615 nm. Therefore it is now possible to monitor changes in amount of 2 different targets at one time in the same well (Figure 1).

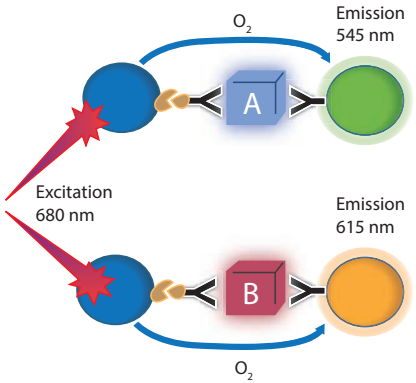


Fig. 1: Dual AlphaLISA principle: Two AlphaLISA's at once For each protein target one antibody was conjugated to an acceptor bead and a 2nd biotinylated antibody was used. Streptavidin coated donor beads were added subsequently.

Both emission wavelengths can be measured simultaneously with the AlphaLISA SDE optic module.

Materials & Methods

- Streptavidin Alpha donor beads
- AlphaLISA® Acceptor beads
- AlphaPLEX™ 545 Acceptor beads
- Purified proteins (Target 1 & 2), Antibodies
- Packard proxiplate F, 384 well, black
- PHERAstar *FSX*

Antibody pairs were selected for each of two protein targets. For each protein target, one of the paired antibodies was conjugated to either AlphaPLEX™ 545nm acceptor beads (for protein target 1) or AlphaLISA® acceptor beads. The other antibody of each pair was biotinylated.
For test purposes two fold serial dilution of protein target 1 were prepared from a starting concentration of 256 µg/mL. Plates were prepared mixing the varying concentrations with either buffer or 128 µg/mL of target protein 2. A similar dilution series of target protein 2 was prepared and plated with either buffer or 128 µg/mL of target protein 1.
A mixture containing acceptor beads and biotinylated antibodies for both target proteins was prepared and added to the reaction plates. After an incubation period of approximately one hour, a solution of donor beads was added to the reaction plates. Following donor bead addition, reaction plates were incubated for approximately one hour at room temperature before being read on the PHERAstar *FSX* using either a standard AlphaLISA module (Em: 615 nm) or an AlphaLISA SDE module (EmA: 615 nm, EmB: 545 nm).

PHERAstar FSX Instrument settings

Focal height	8.0 mm
Aperture spoon	384 well
Settling time	0.1 sec
Excitation time	0.26 sec
Integration start	0.28 sec
Integration time	0.12 sec

Results & Discussion

We first felt it important to verify that well characterized AlphaLISA® results would not be compromised when the AlphaLISA® SDE module is used. The performance of 2 modules in the detection of protein target 2 on separate plates is compared in Figure 3 and Table 1.

Rev. 01/2016
Keywords: **Acceptor beads, AlphaLISA, AlphaPLEX Tb545, Europium, Simultaneous Dual Emission SDE**

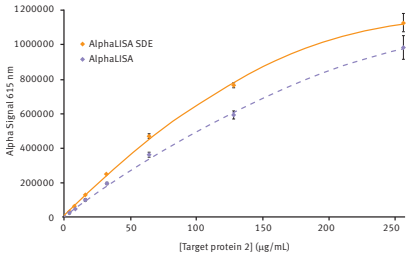


Fig. 3: Comparison of AlphaLISA and AlphaLISA SDE module performance. Alpha signal is plotted vs. the different concentrations of target protein 2 present. Average signal from AlphaLISA (◆) AlphaLISA SDE (◆) modules could be plotted using a 2nd order polynomial function with R2 values of 0.9986 and 0.9991 respectively. Error bars indicate standard deviation (n=8).

Table 1: Assay parameters obtained either with the AlphaLISA standard optic module or with the AlphaLISA SDE optic module

	AlphaLISA®	AlphaLISA® SDE
signal/blank*	138.1	124.8
z-prime**	0.797	0.863

As can be seen the SDE module exhibits comparable performance to the traditional AlphaLISA® module.

Next we wanted to investigate any effect on detection that might occur if a second signal is present in the well. To that end dilutions of target protein 1 were plated in the presence of target protein 2. Plates were subsequently read with the AlphaLISA SDE module. As can be followed in Figure 4 despite a high level of signal in the 615 channel the detection of signal in the 545 is uncompromised.

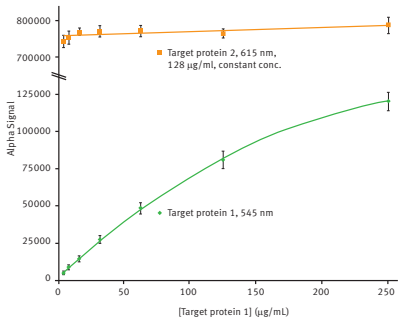


Fig. 4: Comparison of AlphaLISA SDE module signal detection for 545 nm and 615 nm emission. Average 545 nm Alpha signal (◆) is plotted vs. the different concentrations of target protein 1 present using a 2nd order polynomial function (R2 = 0.9997). Average 615 nm Alpha signal (■) is plotted for comparison. Error bars indicate standard deviation (n=8).

Similarly we wanted to assess any possible effect that the presence of 545 nm signal may have on the detection in the 615 nm channel. The results in Figure 5 indicate that presence of 545 nm signal does not impact detection of 615 nm signal.

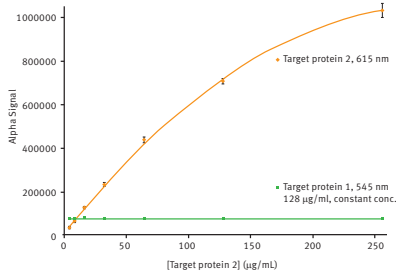


Fig. 5: Comparison of AlphaLISA SDE module signal detection for 615 nm and 545 nm emission. Average 615 nm Alpha signal (◆) is plotted vs. the different concentrations of target protein 2 present using a 2nd order polynomial function (R2 = 0.9997). Average 545 nm Alpha signal (■) is plotted for comparison. Error bars indicate standard deviation (n=8).

Conclusion

The PHERAstar *FSX* proves to be an excellent reader to perform detection of Alpha signals at 615 nm and 545 nm simultaneously without compromising data quality.

* Signal of protein at 256 µg/ml divided by no protein control
** based on data for protein at 256 µg/ml as positive control and no protein control as negative control

PHERAstar® *FSX*
PHERAstar® *FS*

A fully automated kinetic solubility screen in 384-well plate format using nephelometry

Caroline Green, Seona McKee and Ken Saunders, Automation Team, Department of Drug Metabolism
Pfizer Global Research and Development, Sandwich Laboratories, Sandwich, Kent CT13 9NJ, UK.

- **Nephelometry as a rapid, reliable and low cost method for solubility screening in 384-well plate format**
- **Kinetic solubility determination of 24 compounds in 75 min**
- **Special fit method for solubility curve**

Introduction

Within the drug discovery industry there is a growing trend towards measuring ADME and physical chemical properties for larger numbers of compounds at an earlier stage, and at a higher throughput, in an attempt to highlight potential ADME issues and to reduce attrition. Solubility is one of the most important properties of a compound and recognising solubility issues at an early stage is invaluable. Not only are low solubility compounds more difficult to develop, obtaining reproducible data for ADME screens such as Caco-2 and lipophilicity is also more time-consuming and costly. Therefore, a rapid, low cost method for determining solubility prior to running the more costly ADME screens is a useful tool.

Laser nephelometry has been shown to be a reliable technique for the measurement of kinetic solubility in 96-well plate format. Laser nephelometry is the measurement of forward scattered light when a laser beam is directed through a solution. The more particulate there is in the solution, the greater the amount of forward scattered light (measured as counts). This work shows how this technique has been advanced into a fully automated and rapid kinetic solubility screen in 384-well plate format.

Materials & Methods

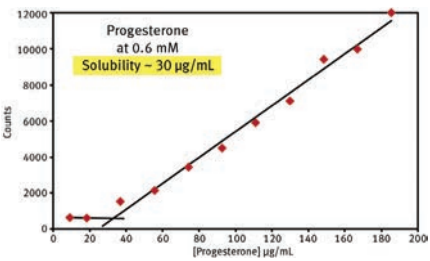
- A liquid handling robot has been integrated with a BMG LABTECH NEPHELOstar® *Plus* to produce a fully automated kinetic solubility screening system for discovery compounds
- The robot is fitted with 1 mL syringes, fixed tips and a ROMA arm.
- The liquid handling, pipetting volumes, plate type, plate reading, time scheduling and processing software have been optimised resulting in an analysis and data processing time of 75 minutes for 24 compounds (8 compounds in quadruplicate per 384-well plate).
- This system has been validated with 38 commercial and in-house compounds.

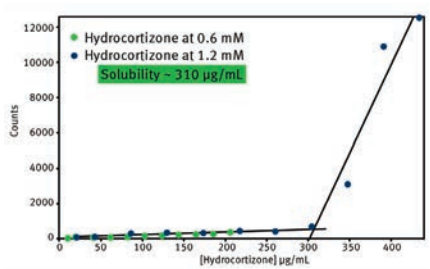
Experimental

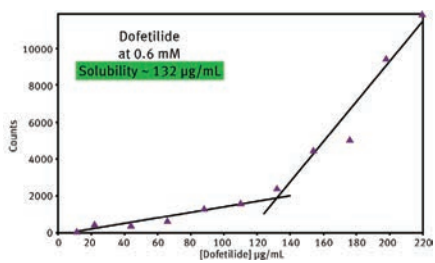
- Stock solutions were prepared at 0.6 mM or 1.2 mM in 5% DMSO: 95% PBS buffer manually.
- For a single batch of 24 compounds (3 plates), the stock solutions were diluted to decreasing molarity across the plate with 5% DMSO: 95% PBS buffer by the robot.
- The optimum net volume for all samples was found to be 100 µL. This is the maximum volume to fit into a 384-well plate, whilst minimising pipetting errors at low volumes (< 5 µL).
- Each plate was read vertically, with a gain of 100 and a laser intensity of 90% to produce raw data of counts per well.
- All raw data were processed using BMG LABTECH MARS data analysis software.

Results & Discussion

The following graphs from below (figure 1) show: mean counts (n=4) plotted against concentration for progesterone, hydrocortizone, dofetilide and paracetamol.







Rev. 10/2004
Keywords: **Dofetilide, Hydrocortizone, Paracetamol, Progesterone, Solubility**

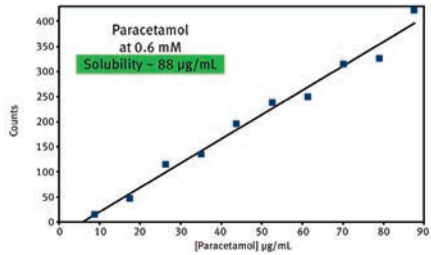


Fig. 1: Kinetic solubilities for 4 compounds evaluated from the fitted mean counts (n=4).

For the first 3 compounds there is a dramatic increase in counts which corresponds to the compound precipitating out of solution. Two linear lines are fitted to the data and the point at which they cross is taken as the kinetic solubility. The plot for paracetamol shows no point of precipitation which indicates complete solubility over the concentration range covered.

Hydrocortizone has been chosen as a control compound to assess the batch to batch variability of the system. Figure 2 shows the results for hydrocortizone run over 6 different days (n=14). The mean result is (289 ± 14) µg/mL. In the future, the result for hydrocortizone run in each batch will have to fall within this range for the batch to be accepted. The batch to batch variability of the system is 5%.

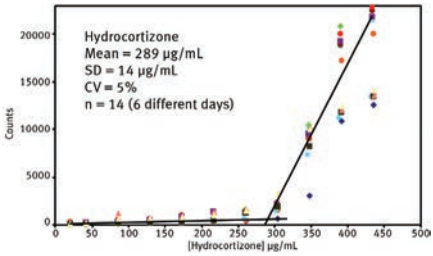


Fig. 2: Batch to batch variability for hydrocortizone as a control compound. Results over 6 different days (n=14) are shown.

Table 1: Comparison of this work with results found in literature.

Compound	This work[a] µg/mL	Literature[b] µg/mL
Hydrocortizone	289	>181
Tyrosine	63	91
Propanolol	>156	41 (10)[c]
Paracetamol	>88	Na
Progesterone	30	20
Dofetilide	132	Na
Acetazolamide	>118	>111
Nitrofurazone	>119	>99
Theobromine	80	90
Ibuprofen	>123	52 (1)[c]

[a]Measured solubility in 384-well plate format at pH 7.4.
[b]Measured solubility in 96-well plate format at pH 7.4.
[c]Measured solubility in 96-well plate format at pH given in parentheses.

38 compounds have been run on the system described here as part of the validation. It was possible to obtain measured solubilities for 35 out of the 38 compounds. A non-result was due to the plots of mean counts against concentration being too scattered to fit any reasonable line through. 10 of the compounds measured in this work have been measured previously using nephelometric techniques in 96-well plate format. Table 1 shows that the results using the fully automated 384-well plate system and the manual 96-well plate method compare favourably.

Conclusion

- This novel, fully automated kinetic solubility screen in 384-well plate format can be used to analyse 24 compounds in 75 minutes with a batch to batch variability of 5%.
- The screen can be used to measure and rank the kinetic solubility of approximately 90% of discovery compounds submitted to the screen.
- Results will be reported as measured values and flagged as green for soluble (>100 µg/mL), amber for partially soluble (15 - 100 µg/mL) and red for poorly soluble (<15 µg/mL) compounds.
- The system can be easily adapted to work with alternative buffers and pHs.

NEPHELOstar® *Plus*

Monitoring of microbial growth curves by laser nephelometry

Sven Brand and Christian Rückert
Department of Genetics, University of Bielefeld

- **Microbial growth monitoring for 48 hours with great reproducibility**
- **Nephelometry is clearly superior to turbidimetry regarding sensitivity**
- **Good correlation between absorbance and nephelometry measurements**

Introduction

Different analytical approaches in clinical immunology and drug discovery take advantage of two closely related techniques based on light scattering.
Turbidimetry is the measurement of light transmitted through a suspension of particles. It requires relatively higher concentrations of particles and obeys Beer's Law.
In contrast, nephelometry is a direct method of measuring light scattered by particles suspended in solution at right angles to the beam, or preferably, at a forward angle. In dilute solutions, where absorption and reflection are minimal, the intensity of the scattered light is a function of the concentration of scattering particles.
The most common application of laser-based nephelometry in microplate format is the fully automated solubility screen in HTS laboratories. Determining aqueous compound solubility has become an essential early measurement in the drug discovery process to avoid time-consuming and costly ADME screens of low solubility compounds.
In clinical chemistry, nephelometry is used to determine serum immunoglobulin (IgA, IgG, IgM), complement components (C3, C4), acute phase reactant proteins (CRP, transferrin), albumin and a-1-antitrypsin. Protein precipitation of globular proteins refers to the formation of protein aggregates by adding salt (ammonium sulphate), organic solvent (acetone), organic polymer (PEG) or trichloro-acetic acid. In contrast, immunoprecipitation allows for a given protein to be precipitated selectively via an antibody-antigen reaction.
In organic chemistry, nephelometry is used to quantify macromolecules, e.g. monitoring of a polymerisation reaction.

Materials & Methods

As an alternative to the common cuvette based instruments BMG LABTECH offers the only laser-based microplate nephelometer, the NEPHELOstar® *Plus*, which detects particulate matter within microplate wells via forward light scattering (optical design is described in figure 1).
The light source of the NEPHELOstar *Plus* is a red laser diode (633 nm) which offers adjustable intensity and beam diameter to reduce meniscus effects and optimized sensitivity allowing to measure even in 384-well plate format. Instrument flexibility is further enhanced by two built-in reagent injectors, precise temperature control, multimode shaking capabilities, automatic gain adjustment, and a robotic plate carrier.

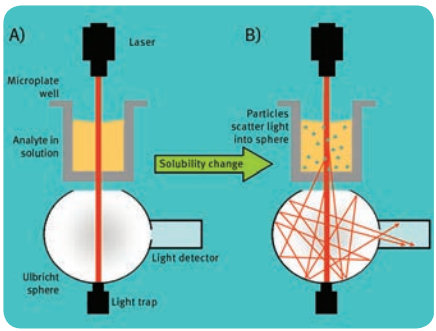


Fig. 1: Schematic diagram of the measurement principle of the NEPHELOstar *Plus*: A clear solution with minimal scattering results in low signal (A). A solution with particles scatters light and results in higher signal (B).

A fast and sensitive method of analyzing growth phase regulation of the Corynebacterium glutamicum wild type and different deletion mutants by continuous monitoring of microbial growth on a medium supplemented with different sulphur sources is described below. Corynebacterium glutamicum is a Gram-positive, non pathogenic and fast growing soil bacterium which is used for industrial amino acid and vitamin production. Cells were precultured on modified minimal medium E (MMES) supplemented with different suitable sulphur sources at 30°C in a rotary shaker (300 rpm), and the optical densities at 600 nm of these cultures were measured. These precultures were used to inoculate 100 µL of growth media (MMES + 0.1 mM sulphur source, start OD of 0.01) using the microplate Cellstar, 96 Well Suspension Culture Plate (GreinerBioOne). To avoid evaporation and condensation the plates were sealed with transparent, hydrophobic and gas permeable plastic films (Breathe-Easy, Roth) during incubation and measurement.
The growth curves were recorded by monitoring the turbidity of the cultures using the NEPHELOstar *Plus*. The laser intensity was adjusted to 50% and the laser beam focus to 2.5 mm. Incubation temperature was 30°C, and between measurement cycles the plate was orbitally shaken with a shake width of 3 mm.

Results & Discussion

In earlier experiments the comparability of the data obtained by optical density methods was assured using a transmission reader with data obtained by measuring the scattering of light using a nephelometer. A serial dilution of the C. glutamicum culture was made and

Rev. 07/2004
Keywords: ***Corynebacterium glutamicum*, Microbial growth, Nephelometric principle, OD_{600}, Turbidity**

turbidity determined by measuring both the optical density and the forward scattered light. Fig. 2 shows a good correlation between the two methods, which is almost linear up to 4 OD.

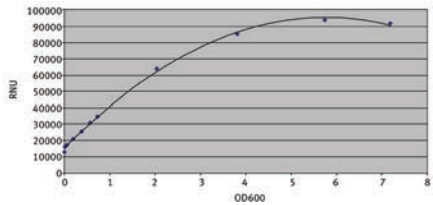


Fig. 2: Correlation of the OD values (absorbance at 600 nm, BMG LABTECH reader) with the nephelometric approach (NEPHELOstar, BMG LABTECH), using a serial dilution of the C. glutamicum culture.

The great reproducibility of the nephelometric assay is demonstrated in figure 3, which shows four independent growth curves of a C. glutamicum culture. In this graph six replicates were used for each curve plotted against time and including the mean of these four growth curves.

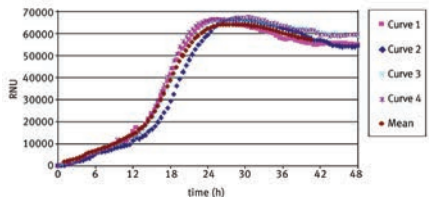


Fig. 3: Four independent growth curves of a C. glutamicum culture monitored with the NEPHELOstar.

Finally, figure 4 demonstrates a concrete biological application of nephelometry in bacterial growth regulation. It shows the growth curves of the C. glutamicum wild type and a deletion mutant, which has a deleted gene for a regulatory protein. Both strains are cultured on minimal medium supplemented with different sulphur sources (S1, S10, S11, and S24). The plotted growth curves show that the deletion mutant, compared to the wild type, grows better on S1 but worse on S10 and no more on S24.

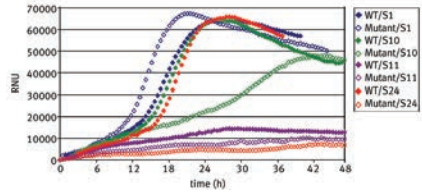


Fig. 4: Growth curves of the C. glutamicum wild type and a deletion mutant cultured on minimal medium supplemented with different sulphur sources.

Conclusion

The described application reveals that laser nephelometry is a reliable technique for monitoring microbial growth besides the classical applications like compound solubility testing and immunoprecipitation. Studies show that the nephelometric assay, compared to the turbidimetric assay, is not only comparable, but clearly superior regarding sensitivity. The key advantage of nephelometry is the ability to detect scattered light, even if the concentration of scattering particles is very low, which is the case during the lag phase and beginning of the log phase. Using the NEPHELOstar *Plus*, instead of a traditional transmission reader, this early part of the growth curve can be monitored much more accurately.

NEPHELOstar® *Plus*

Measurement of Rheumatoid Factor by NEPHELOstar® *Plus* microplate reader

Lisa Mack and Anne Barton
ARC-Epidemiology Unit, Stopford Building, University of Manchester, UK

- **NEPHELOstar® *Plus* provides higher throughput and cost effectiveness for the detection and quantification of rheumatoid factors**
- **Results are concordant with large scale IMMAGE® Immunochemistry System**

Introduction

Rheumatoid Factors (RF) are immunoglobulins which react to antigenic sites on the Fc region of human or animal immunoglobulin G (IgG). The main isotype is IgM although others occur (IgA, IgD and IgG). RF are found more commonly in patients with rheumatoid arthritis (RA) than the general population but can be found in patients with other connective tissue diseases, following infection and in a number of other diseases such as sarcoidosis and liver disease. However, presence of RF is one of the classification criteria for RA and presence of RF is associated with a poorer prognosis such as increased risk of developing erosive disease.

RF may be measured by a number of methods. These include the particle agglutination test, which relies on the agglutinating properties of IgM class of RF. IgG, usually either human or rabbit, is bound to a carrier such as latex and the presence of RF is detected by flocculation. Doubling dilutions of the serum until the flocculation can no longer be visualized allows a semi-quantitative measurement of antibody titre. However, the assay requires large amounts of serum, is time-consuming and observer dependent.

An alternative method of measuring RF uses nephelometry. Patient serum is added to a microplate containing a fixed amount of antigen (IgG). Any RF present will form an antibody-antigen complex with the IgG and the concentration of the RF antibody can be determined by light dispersion. Potential advantages offered by this system are better throughput, objective reading on a single sample dilution, use of smaller volumes of serum and cost effectiveness.

In clinical diagnostic settings, measurement is usually performed using an IMMAGE® Immunochemistry Systems but such platforms are expensive to purchase and maintain. This application note investigates a method for testing RF using the BMG LABTECH NEPHELOstar *Plus* microplate reader.

Materials & Methods

Principle

Nephelometric testing of rheumatoid factor levels is based upon antigen-antibody reactions. RF binds to IgG to form immune complexes. As the number of immune complexes grows, the solution becomes more visibly "cloudy". The NEPHELOstar *Plus* microplate reader passes a light beam through the well in order to measure the turbidity of the sample. The more RF present, the more immune complexes are formed and the more light scattering will occur. The amount of light scattered is directly proportional to the number of immune complexes and therefore RF particles suspended in the sample.

Materials

- NEPHELOstar *Plus* plate reader and software (BMG LABTECH)
- RF-PAIA kit, including set of 5 calibrants, reagent and buffer (Orion Diagnostica, Finland)
- 96-well clear microplates (Bibby-Sterilin Ltd, UK)

Operating Procedure

1. Plate out 10 µL of undiluted calibrators 1 to 5, a known RF positive and negative serum and the serum samples to be tested.
2. Add 280 µL of RF buffer.
3. Pipette up and down gently to mix the solution, whilst trying to avoid bubbling*.
4. Insert the plate into the NEPHELOstar *Plus* microplate reader and adjust the settings for plate mode kinetic as shown:
   - Gain: 85
   - Pos. delay: 0.1 s
   - No. of cycles: 2
   - Meas. start time: 0 s
   - Meas. time/well: 1 s
   - Laser intensity: 50 %
   - Beam focus: 2 mm
   - Shaking time: 20 s
   - Shaking width: 4 mm
   - Shaking before each cycle
5. Begin the test. Cycle 1 will shake the plate and take the background reading.
6. When prompted, remove the microplate and add 10 µL of reagent to each well, pipetting up and down to aid mixing*.
7. Insert the plate again and press continue. Cycle 2 will shake the plate once more, and take the final reading.

* Mixing using a pipette should be performed with care. Generation of bubbles during mixing will interfere with the assay and may lead to inaccurate results.

Results & Discussion

80 samples with five calibrants and a positive and negative control can be measured in approx. 2 minutes. Including pipetting of all solutions (multichannel for buffer, all others singly pipetted), the run takes approximately 2 hours.

Results obtained for serum samples tested by the NEPHELOstar *Plus* microplate reader were compared with those from a Beckman Coulter IMMAGE® Immunochemistry System using the same serum samples (Fig. 1). Regression analysis showed that the correlation between the readings was 94.1%.

Rev. 08/2005
Keywords: **Antibody, IgG, IgM, Immunoglobulins, Rheumatoid factor, Serum**

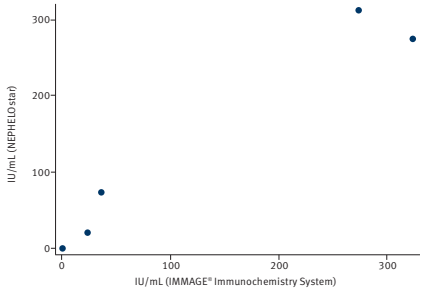


Fig. 1: Measurement of five RF-PAIA calibrants on the IMMAGE® Immunochemistry System vs NEPHELOstar *Plus* microplate reader.

Conclusion

Through this independent analysis, we have shown that the NEPHELOstar *Plus* plate reader can be used to accurately determine both RF antibody status and titre, which is critical to the diagnosis of rheumatoid arthritis and sub group definition of juvenile idiopathic arthritis. Thus this RF assay is a viable and inexpensive assay that can be used in medium to high throughput labs.

NEPHELOstar® *Plus*

Nephelometric monitoring growth of *Candida albicans* using BMG LABTECH's NEPHELOstar® *Plus*

U.-C. Hipler,
Universitaetsklinikum Jena, Teilkoerperschaft der Friedrich-Schiller-Universitaet Jena

- **Laser nephelometry used for fungal growth determination and for measurement of drug solubility**
- **Monitoring the growth of *Candida albicans* in presence of antifungal agents**
- **Phase solubility diagrams of potential drug presented**

Introduction

Cyclodextrins (CD) have become useful pharmaceutical excipients, due to their potential to form inclusion complexes with appropriately sized drug molecules. The resulting complexes generally offer a variety of physico-chemical advantages over the free drug, including increased water solubility, enhanced bioavailability, improved stability, reduced side effects, etc.

Econazole-nitrate (EC) and ciclopirox-olamine (CI) are well known antifungal agents suitable for the treatment of many mycotic infections. Previous studies showed that both dissolution properties and consequently microbiological activities of econazole, with very low water solubility (about 3 µg/mL at 25°C), can be improved by complexation with natural cyclodextrins, particularly with β-CD3-5.
By increasing the water solubility of the drug it should be possible to improve its bioavailability, thus enabling improved oral or topical formulations.

Laser nephelometry has been shown to be a reliable technique for the measurement of drug solubility in plate formats up to 384 well. Laser nephelometry is the measurement of forward scattered light. When a laser beam is directed through a clear solution, the more particles or turbid suspensions (fungi in this study) in the solution, the greater the amount of forward scattered light (measured as units). The energy of the scattered light is directly proportional to the particle concentration in the suspension for up to three orders of magnitude. Herein we describe the use of laser nephelometry to investigate the effects of complexation on the drug antimycotic activity using BMG LABTECH's NEPHELOstar® *Plus*. Furthermore nephelometry was used to prepare phase solubility diagrams and to monitor fungal growth.

Materials & Methods

- *Candida albicans* DSM 11225
- Sterile and clear 96-well plates, Greiner bio-one
- β-Cyclodextrin, Wacker-Chemie
- BMG LABTECH NEPHELOstar *Plus*

Preparation of cultures
C. albicans were grown on SGA (Sabouraud-Glucose-Agar with gentamycin-chloramphenicol from bioMerieux, Germany) at 30°C for 24–48 h. Three to five well-isolated colonies of the same morphological type were selected from an overnight culture using a sterile wire loop and inoculated in 20 mL (SGB). The suspensions were incubated with shaking at 250 rpm/30°C for 24 h. Then the overnight cell cultures were counted using CASY® 1 and adjusted to a final working concentration of 6×105 cells/mL in SGB (Sabouraud-Glucose-Bouillon from Oxoid Ltd, UK).

Preparation of antifungal agents
Both EC and CI were independently dissolved in a mixture of chloroform/methanol 1:1 to achieve a final stock solution containing 20 mg/mL of antifungal agent. The stock solution of EC was diluted with SGB and adjusted to be 1.25–100 µg/mL while as for CI; it was in the range of 1.25–10 µg/mL. All solutions were stored at –80°C until used.

Preparation of the inclusion and antifungal complexes
A solution of EC was prepared by dissolving it in a chloroform/methanol mixture 1:1. CD was dissolved in hot water at 85–90°C. Equimolar amounts (1:1 molar ratio) of EC and CD solutions were mixed together with stirring for 30 min at 85–90°C. By cooling, crystallization of the complex was obtained. The complex was filtered using G3 filter and kept in a desiccator overnight. On the other hand, the second complex between CI and CD was also prepared according to the previously mentioned method, in which methanol was used as a proper solvent for CI. Moreover, a molar ratio of 1:2 of CI:CD was also used. The antifungal complex of CD–EC was prepared in a concentration range of 12.5–100 µg/mL using DMSO as a solvent, while the CD–CI was prepared in a concentration range of 150–400 µg/mL using distilled water.

Phase solubility studies
In this experiment, both of drugs and complexes were diluted in DMSO. Then the drug and complex solutions were independently pipetted into PBS-buffer with a concentration of DMSO of 1–5 vol.%. All samples were measured on the NEPHELOstar *Plus* at 30°C, with an integration time of 0.1 s, so that a plate (96 samples) could be scanned in ~68 s. A gain of 122 and a laser intensity of 1 % were set to allow direct comparison of all results. All raw data were processed using the BMG LABTECH MARS data analysis. The scattered light will remain at a constant intensity until precipitation occurs. At that point it will increase sharply.

Results & Discussion

Phase solubility diagrams
Solubility diagrams were monitored using laser nephelometry which determines the solubility of potential drug candidates supplied as dimethyl sulfoxide (DMSO) solutions in 96-well plates (Figure 1).

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Keywords: ***Candida albicans*, Ciclopirox-olamine, Cyclodextrins, Econazole-nitrate, Microbial growth**

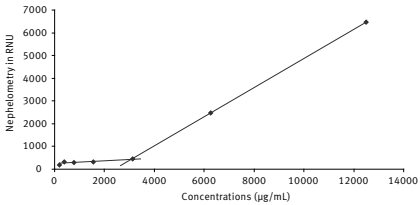

Fig. 1: Solubility diagram of CD-Econazole-nitrate complex.

In the absence of CD, the solubility of EC is determined to be ~1.3 mg/mL (graph not shown), while in the presence of CD the solubility increased up to ~3.1 mg/mL for CD–EC complex.

The solubility results for ciclopirox-olamine (CI) differ from the other antifungal agent.

In the absence of cyclodextrin, the solubility of CI is determined to be ~1.2 mg/mL (graph not shown). Linear line was obtained for CD–CI complex and no point of precipitation is found which indicated complete solubility over the concentration ranges (Figure 2).

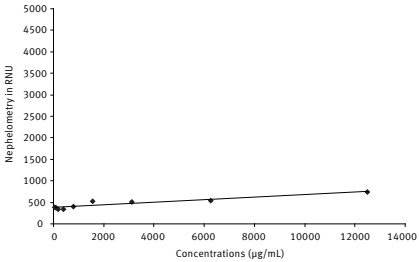

Fig. 2: Solubility diagram of CD-Ciclopirox-olamine complex.

Influence of complexed antifungal agents on the growth of *Candida albicans*

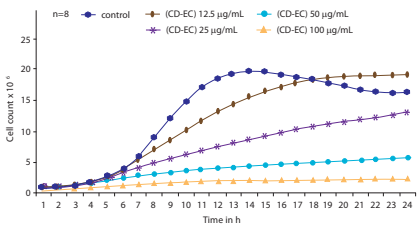

Fig. 3: Influence of ciclopirox complex (CD-CI) on the growth of *Candida albicans*.

In contrary to CD-EC the complexed ciclopirox was less effective (Figure 3). At a concentration of 200 µg/mL of the complex, cells were inhibited and at a concentration of 300 µg/mL of the complex, cells were killed. The CD-CI complex was also less effective compared to the free CI that caused cell death already at 10 µg/mL.

Conclusion

This study has proven that laser nephelometry in a 96-well microtiter plate can be used as a method for the rapid determination of the solubility of potential drug compounds.

Laser nephelometry can distinguish between the concentration at which the drug just goes into or just comes out of solution. On the other hand, this technique can be used efficiently for monitoring and evaluating the growth of microorganisms like fungi or bacteria.

NEPHELOstar® *Plus*

96-well nephelometric assay to detect calcification propensity of serum samples

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- **Cardiovascular diseases are related to vascular calcification**
- **Fetuin-A is a strong calcification inhibitor**
- **Assay can be applied to serum samples from patients and healthy individuals**

Introduction

Calcium ions (Ca^{2+}) and phosphate are used by the human body to build the structural material of bone and teeth. The resulting crystalline calcium phosphate is the product of the calcification process. Concentrations of Ca^{2+} and phosphate are close to supersaturation in most tissues and body fluids, and as a result precipitation could happen anywhere in the body. In healthy people this is usually not the case and so it is comprehensible that the calcification process must be very strictly regulated. If this regulation is not working properly, calcification can also take place in soft tissues or blood vessels, leading e.g. to cardiovascular diseases, the most predominant cause of death all over the world.

The serum protein fetuin-A is a potent calcification inhibitor. Together with additional blood components, fetuin-A will prevent that Ca^{2+} and phosphate precipitate despite supersaturation. Small colloidal protein-mineral complexes are generated instead. These primary calciprotein particles (CPP) will spontaneously convert to crystalline secondary CPP while changing in shape and particle diameter. The transition happens in a timed and coordinated manner and is thought to reflect the intrinsic inhibitory calcification propensity of a given fluid. In this application note we describe a test that measures the transition from primary to secondary CPP in serum samples. This label-free 96-well plate-based assay measures the conversion of primary to secondary CPP by detecting the changes in laser light scattering associated with it. We used a NEPHELOstar® *Plus* microplate reader from BMG LABTECH.

Assay Principle

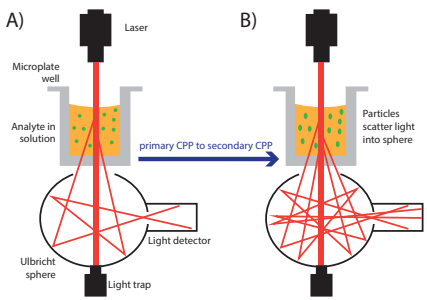

Fig. 1: Nephelometric assay principle.

A laser-generated light beam is passed through a sample. The particles in solution will scatter light depending on particle size and/or shape. Primary CPP and secondary CPP have different shapes and particle diameters enabling the NEPHELOstar *Plus* to measure both states. The forward scattered light is detected at an angle up to 80 degrees.

Materials & Methods

- NEPHELOstar *Plus*, BMG LABTECH
- Liquidator96 bench-top pipetting system, Mettler Toledo
- clear 96-well plates from Brand
- 96-well adhesive plastic cover sheets from Carl Roth
- all chemicals from AppliChem, Sigma or Roth

Serum Samples

Serum samples from healthy volunteers as well as serum samples from hemodialysis patients were assayed. It has been shown that patients that undergo hemodialysis have an increased risk for accelerated vascular and soft tissue calcifications. Mouse sera was prepared from DBA/2 fetuin-A-deficient (-/-), heterozygous (+/-) and wild-type (+/+) mice.

Microplate Preparation

All liquids were pipetted using the Liquidator96 benchtop pipetting system in the following order (volumes for one well)

- 20 µl NaCl solution (140 mM NaCl)
- 80 µl serum
- Mix 1 minute using a vibrating shaker
- 50 µl phosphate solution (19.44 mM Na_2HPO_4+4.56 mM NaH_2PO_4+100 mM Hepes+140 mM NaCl pH-adjusted with 10 M NaOH to 7.40 at 37°C)
- Mix 1 minute using a vibrating shaker
- 50 µl calcium solution (40 mM $CaCl_2$+100 mM Hepes+ 140 mM NaCl pH-adjusted with 10 M NaOH to 7.40 at 37°C)
- Mix 1 minute using a vibrating shaker
- Cover 96-well plate using adhesive sheets

NEPHELOstar instrument settings

The plate was put into the NEPHELOstar and read using the following parameters:

Plate mode kinetic	
No of cycles:	200
Measurement time:	1.5 seconds
Cycle time:	180 seconds per cycle
Positioning delay:	0.1 seconds
Laser beam focus:	1.5 mm
Laser intensity:	50 %

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Keywords: **Calcification, Calcium, Cardiovascular, Hemodialysis, Serum**

Total assay run time was 10 hours, but some measurements were also longer. To minimize temperature fluctuations during measurements, the internal NEPHELOstar temperature control was turned off and all measurements took place in a thermostated room at 34.5°C, leading to an internal temperature of 36.5-37°C in the NEPHELOstar.

Results & Discussion

Using three-dimensional dynamic light scattering (3D-DLS), we determined the hydrodynamic radius Rh for primary CPP and secondary CPP. CPP diameters were measured both in test solutions containing fetuin-A, phosphate and Ca^{2+} as well as in serum samples. The resulting diameters were quite similar (primary CPPs were between 60 and 75 nm whereas secondary CPP were between 120 and 150 nm) but showed significantly different transition times (delayed in serum samples). We concluded that the delay of the conversion step can be related to the stability of primary CPP and that measuring this step will help to determine the inhibitory potency inherent in serum samples. Optimization of assay and all further measurements were done with the NEPHELOstar. Fig. 2 shows a measurement over time while primary CPP convert to secondary CPP.

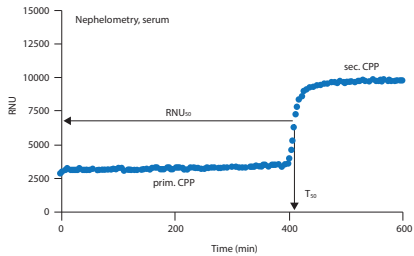


Fig. 2: Primary to secondary CPP transformation step over time in the presence of medium.

After measurement, data were exported to Excel and GraphPad prism for data evaluation. The one-half maximal transition time (T_{50}) and the one-half maximal nephelometric units (RNU_{50}) were calculated (Fig. 2). For assay validation, mouse and human samples were measured. Figure 3 shows that the assay could discriminate between mouse sera derived from wild type and fetuin-A-deficient sera. The nephelometric results agreed well with the calcification load observed of these mice.

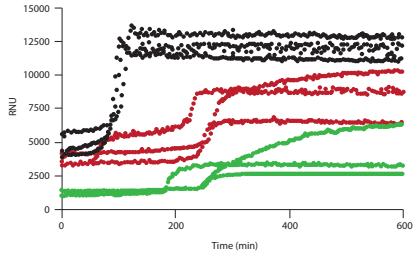


Fig. 3: Nephelometry assay using sera from adult 10- to 16-month-old noncalcifying wild-type DBA/2 mice (green), noncalcifying heterozygous fetuin-A+/2 knockout mice having half-normal serum fetuin-A (red), and heavily calcifying fetuin-A–deficient homozygous fetuin-A2/2 knockout mice (black).

Human serum samples were obtained from healthy donors and hemodialysis patients. Fig. 4 shows the results for both groups.

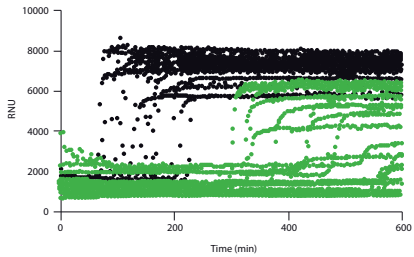


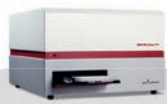
Fig. 4: Nephelometry assay with sera from 20 hemodialysis patients (black) and 20 healthy volunteers (green).

Again the test was able to distinguish between the two donor groups indicating the utility of the test for measurement of human serum samples. Our results demonstrate that the nephelometer test is useful to measure calcification propensity in body fluids with reasonable sample throughput.

Conclusion

Our test is a novel 96-well nephelometer-based assay which measures the overall calcification risk in serum. As calcification is an often observed process in a lot of diseases this assay may be used in clinical and basic research. The routine clinical use of the test requires evaluation in a prospective study.

Patent pending. In case of questions and to avoid IP right infringements, please contact info@calcisco.com



NEPHELOstar® *Plus*

Application notes by number

Keywords

BMG LABTECH can not be held liable for any mistakes or errors in this application notes binder pertaining to product descriptions or specifications.

Assays by company

Company	Page	Assay	AN
Biocolor	29	Cell-APOPercentage™ Apoptosis Assay	228
Beckman Coulter	199	Lumigen SPARCL™ Assay	282
BellBrook Labs	135	Transcreener® ADP2 FI Assay	274
	221	Transcreener® ADPs FP Assay Transcreener® GDP FP Assay Transcreener® UDP FP Assay Transcreener® AMP/GMP FP Assay	225
	225	Transcreener® ADPs FP Assay	269
	255	Transcreener® ADP2 FP Assay Transcreener® ADP2 FI Assay Transcreener® ADP2 TR-FRET Assay	291
Cayman Chemical	253	Prostaglandin D Synthase FP-Based Inhibitor Screening Assay Kit	285
CellAura	103	Adenosine and dopamine fluorescent ligands	227
Cell Biolabs	143	OxiSelect™ Cellular Antioxidant Activity Assay Kit	280
Cisbio Bioassays	185	IP-One HTRF® Assay Kit	137
	187	HTRF® Cortisol kit	154
	191	IP-One HTRF® Assay Kit Cyclic AMP cell-based Assay Kit	209
	195	IP-One HTRF® Assay Kit	222
	201	HTRF® H3K9 mono-methylation detection reagents	249
	205	IP-One HTRF® Assay Kit	273
	227	EPIgeneous™ Methyltransferase Assay Kit	279
	229	Tau aggregation assay kit	286
	235	Development of a Rapid HTRF Insulin Assay	294
CycLex	147	SIRT1 fluorimetric assay kit	292
DiscoverX	55	ADP Hunter™	141
	149	HitHunter™ cAMP assay	134
	151	HitHunter™ cAMP assay	143
	165	PathHunter® GPCR and Kinase Assays	229
	213	HitHunter® IP3 FP Assay	142
Enzo® Life Sciences	172	Leading Light® Sclerostin-LRP Interaction Screening System	255
Gentronix	163	BlueScreen™ HC	218
Greenovation	149	Moss cells as expression system for biopharmaceuticals	293
Knight Scientific	137	ABEL® Antioxidant Assay	108
	141	ABEL® Antioxidant Assay	112
LGC Genomics	131	KASP Genotyping Assay	265
Lonza	145	MycoAlert™ Mycoplasma Detection Kit	130
	147	PKLight™ Protein Kinase Assay Kit	131
	31	Kinetic-QCL™ Kinetic Chromogenic LAL Assay	237
	183	Clonetics™ primary sensors	275
	197	MycoAlert™ Mycoplasma Detection Kit	281
Luxcel Biosciences	193	pH-Xtra™ Glycolysis Assay	219
	197	MitoXpress™ Xtra – Oxygen consumption assay	223
	199	MitoXpress™ Xtra – Oxygen consumption assay pH-Xtra™ Glycolysis Assay	248
	231	MitoXpress®-Intra	290

Millipore	77	Amplifluor® SNP Genotyping System	187
MorphoSys	83	AlamarBlue® cell proliferation assay	195
PAIA Biotech	145	Competitive human IgG Fc kit	288
PerkinElmer	227	IgG AlphaLISA® Detection Kit	190
	229	AlphaScreen® SureFire® ERK1/2 Phosphorylation Assay Kit	210
	231	AlphaLISA® Human TNF-α Research Immunoassay Kit	260
	233	AlphaScreen® P-Tyr-100 (Phosphotyrosine) Assay Kit	270
	265	AlphaLISA® Acceptor Beads AlphaPLEX™ 545 Acceptor Beads	283
	189	DELFIA® TRF cytotoxicity kit	192
PharmaDiagnostics	41	SoPRano™ Gold Nano Rods	276
Platypus Technologies	89	Oris™ Cell Migration Assay	204
ProAxsis	47	ProteaseTag™ Active Neutrophil Elastase Immunassay	289
Promega	141	RealTime-Glo™ MT Cell Viability Assay CellTox™ Green Cytotoxicity Assay	278
	139	ENLITEN® ATP Assay System	110
	153	P450-Glo CYP1A2 Assay	151
	157	DLR™ Gene Reporter Assay System	172
	161	ADP-Glo™ Kinase Assay	202
	169	HDAC™-Glo I/II Assay	247
	179	CellTiter-Glo® EnduRen™ ViviRen™ Caspase-Glo® Apo-One® CellTiter-Blu®	266
	181	DLR™ Certification Kit	271
	183	pGloSensor™22F cAMP	275
	203	NanoBRET™ Assay	287
TFC Bioscience	93	Fluorosome®-trans-pgp Assay	208
TGR BioSciences	99	ELISA-One™ Assay	213
ThermoFisher	49	Voltage Sensor Probes	123
	69	NanoOrange® Protein Quantitation Kit	166
	91	Fluo-4 Direct™ Calcium Assay Kit	206
	123	Fluo-4 Direct™ Calcium Assay Kit	253
	129	Quanti-iT™ PicoGreen® dsDNA Reagent	261
	167	Pierce™ Luciferase Dual Assay Kit	233
	203	LanthaScreen® Tyrosine Kinase and protein kinase C Assay	268
	215	Predictor™ hERG FP Assay kit	173
TwistDx	73	RPA Technology	176
Sigma-Aldrich	11	Bradford	158

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