Fluorescence Intensity, FRET, and TR-FRET Homogeneous Assay Technologies from Invitrogen™ Validated on BioTek's new Versatile Hybrid Microplate Reader, Synergy™ 4

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Abstract

Homogeneous assays have been used in drug screening and automated systems because they can provide scientific information without lengthy wash steps to remove unbound constituents. Several different fluorescence assay technologies offered by Invitrogen™ can be successfully incorporated as part of biomolecular screens for key drug target classes (e.g. kinases, nuclear hormone receptors, and G-protein coupled receptors). Omnia®, Z'-LYTE® and LanthaScreen™ are biochemical assays which can be utilized to assess kinase biology *in vitro*, and GeneBLAzer® is a cellular reporter-gene assay used to interrogate associated kinase signaling pathways *in vivo*. While these homogeneous assay technologies are all amenable to high-throughput screening campaigns, they employ widely different means of detection, including fluorescence intensity, FRET, and TR-FRET. Some assays use top read measurements, while others require that the signal be measured from the bottom of the microplate.

Because of the wide variety of detection requirements demanded by today's screening assays, it is important that the microplate reader used for detection and measurement be not only sensitive, but also versatile. The Synergy™ 4 Multi-Mode Microplate Reader with Hybrid Technology™ provides optimal sensitivity while at the same time having the flexibility to perform measurements of several different assay technologies. Here we demonstrate the validation of several different fluorescence screening assays from Invitrogen™ on the Synergy 4.

Conclusions

Synergy 4:

- Excellent level of performance on a variety of Invitrogen™ drug discovery assays
- High-performance TR-FRET mode for LanthaScreen™ assays
- Bottom, dual-color FRET measurements for GeneBLAzer® cell-based assays
- Top, dual-color FRET measurements for Z'-LYTE® kinase assays
- Monochromator or filter-based kinetic measurements for Omnia® kinase assays
- Hybrid Technology™ ideal for assay development and screening laboratories
- Capable of Fluorescence Spectral Scans

Gen5 Data Analysis Software:

- Powerful reader control features accommodate a wide variety of assays
- Can perform complex calculations and plot curves, all within a 21 CFR Part 11 compliant-environment
- Exports the raw data to third party software in one mouse click

Introduction

Screening assays come in many sizes and formats depending on the target of interest and the information required. As such there are many different technologies employed to meet these diverse requirements. Some assays can assess biological processes *in vitro* while other do so *in vivo*. In addition assays have different and often demanding detection requirements. Many assays produce a colorimetric change that can be measured by its absorbance. Cell proliferation assays that utilize MTT, XTT or WS-1 generate colored azo-compounds that can be measured at the appropriate visible wavelength. NADH linked enzymatic assays can be assessed by the change in absorbance at 340 nm. Luminescence based assays have been used to assess cell growth via ATP as well as for gene expression studies using Firefly and Renilla Luciferase. Several different fluorescence based assay formats have been used for screening assays. Fluorescence intensity measurements of calcein-loaded cells provide useful information on cell proliferation. Fluorescence resonance energy transfer (FRET) and fluorescence polarization experiments can be used to identify specific binding reactions *in vitro* or *in vivo*. TR-FRET, which is very similar to FRET, takes advantage of the long fluorescence life span of lanthanide molecules to greatly reduce background fluorescence.

Several different fluorescence assay technologies offered by Invitrogen™ can be successfully incorporated as part of biomolecular screens for key drug target classes (e.g. kinases, nuclear hormone receptors, and G-protein coupled receptors). Omnia®, Z'-LYTE® and LanthaScreen™ are biochemical assays which can be utilized to assess kinase biology *in vitro*, and GeneBLAzer® is a cellular reporter-gene assay to interrogate associated kinase signaling pathways *in vivo*. While these homogeneous assay technologies are all amenable to high-throughput screening campaigns, they employ widely different means of detection, including fluorescence intensity, FRET, and TR-FRET. Some assays use top read measurements, while others require that the signal be measured from the bottom of the microplate.

Because of the wide variety of detection requirements demanded by today's screening assays, it is important that the microplate reader used for detection and measurement be not only sensitive, but also versatile. The Synergy™ 4 Multi-Mode Microplate Reader with Hybrid Technology™ provides optimal sensitivity while at the same time having the flexibility to perform measurements of several different assay technologies. Here we demonstrate the validation of several different fluorescence screening assays from Invitrogen™ on the Synergy 4.

Materials

The following parts and reagents were supplied by Invitrogen (Carlsbad, CA):

Z'-LYTE® Kinase Assay Kit (catalog number PV3193)
Omnia® Tyrosine Kinase Assay Kit (catalog number KNZ4031)
Syk kinase enzyme (catalog number PV3857)
LanthaScreen™ Tb-anti-GST Antibody Kit (catalog number PV4216)
CellSensor® irf1-bla TF1 cell line (catalog number K1219)
LiveBLAzer™ FRET Loading Kit (catalog number K1095)
Solution D (catalog number K1156)
Phosphate Buffered Saline without calcium and Magnesium [PBS(-)] (catalog number 14190-136)

Synergy™ 4 Hybrid Reader

Figure 1. Synergy™ 4 Hybrid Multi-Mode Microplate Reader

The Synergy 4 is a microplate reader that provides the combined benefit of bringing performances and technologies usually found on high-end HTS instrumentation to research laboratories, while at the same time delivering flexibility and efficient cost-control to screening laboratories. The patent pending Synergy 4 utilizes Hybrid Technology™ where both monochromator and filter-based wavelength selection for fluorescence are available. Besides having the choice between filters and monochromators for wavelength selection, fluorescence measurements can be made using either a continuous tungsten-halogen lamp or a xenon-flash lamp for a light source. Multiple sets of optics provide optimal performance regardless of the detection technology. Absorbance measurements use a xenon-flash lamp with a dedicated monochromator for wavelength selection, allowing the selection of any wavelength for endpoint or kinetic measures from 200 nm to 999 nm. Fluorescence polarization is accomplished with the use of polarizing filters in conjunction with label specific dichroic mirrors for wavelength specificity. For time-resolved fluorescence measurements, the Synergy 4 integrates a high-energy xenon-flash-lamp with excitation and emission filters and a PMT detector. Luminescence measurements are made using a dedicated liquid-filled optical fiber to capture light along with a low noise PMT.

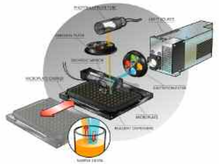

Figure 2. Schematic illustration of the Synergy 4 filter-based fluorescence detection

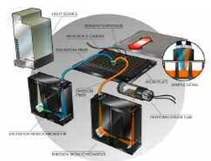

Figure 3. Schematic illustration of the Synergy 4 monochromator-based fluorescence detection

Z'-LYTE® Assay

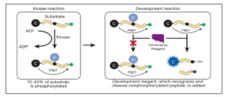

Figure 4. Illustration of the two step Z'-LYTE® assay procedure

Introduction

Z'-LYTE® technology is a homogeneous assay format based on fluorescence resonance energy transfer (FRET), and is a universal platform for screening and profiling protein kinases. The assay uses a peptide substrate labeled with two different fluorophores (coumarin donor and fluorescein acceptor) on each end. These two fluorophores make up a FRET pair, which allows the transfer of energy when both are present on the same molecule. The initial kinase reaction transfers the γ-phosphate of ATP to a tyrosine or serine/threonine residue on the substrate. In a secondary reaction, referred to as the development reaction, a site-specific protease recognizes and cleaves only non-phosphorylated peptide, while phosphorylated substrate remains uncleaved. Uncleaved phosphorylated product will exhibit high FRET acceptor emission, while cleaved peptides will have low FRET acceptor emission. Upon excitation of coumarin at 400 nm, the FRET signal is measured as a ratio between the coumarin donor emission at 445-460 nm and the fluorescein acceptor emission at 520-535 nm.

Methods

20 µL aliquots of Kinase Reaction (Samples and Controls)
Incubate for 30 minutes at RT
Add 10 µL of Development Solution
Incubate for 60 minutes are RT
Add 10 µL of Stop Solution
Read Fluorescence:
Excitation: 400 nm
Emission: 460 nm and 535 nm

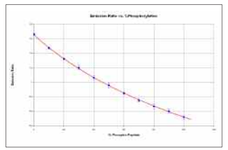

Figure 5. Emission ratio for various degrees of phosphorylation

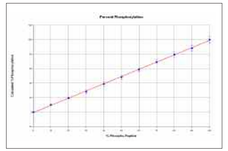

Figure 6. Linear relationship of calculated percent phosphorylation

Eq. 1

$$\% \text{ Phosphorylation} = \left(1 - \frac{(\text{Emission Ratio} \times F_{100\%}\text{-}C_{100\%}}{(C_{0\%}\text{-}C_{100\%}) + (\text{Emission Ratio} \times (F_{100\%}\text{-}F_{0\%}))}\right) \times 100$$

Where:

Emission Ratio	=	Coumarin/Fluorescein ratio of sample wells
$C_{100\%}$	=	Average Coumarin signal of the 100% phosphorylated control
$C_{0\%}$	=	Average Coumarin signal of the 0% phosphorylated control
$F_{100\%}$	=	Average Fluorescein signal of the 100% phosphorylated control
$F_{0\%}$	=	Average Fluorescein signal of the 0% phosphorylated control

Results

- Emission ratio decreased with increasing percentage of phospho-peptide
- Calculated % phosphorylated peptide correction provides linear relationship

Omnia® Assay

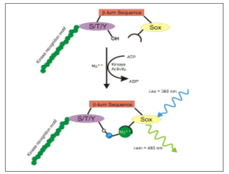

Figure 7. Schematic diagram of the Omnia® Kinase Assay

Introduction

Omnia® kinase assays utilize a fluorescence peptide substrate-based technology for the rapid, homogenous and sensitive real-time detection of kinetic enzymatic activity. The chelation-enhanced fluorophore (CHEF), also referred to as Sox, is an unnatural amino acid that can be incorporated into substrate peptides using standard solid-phase peptide chemistry. Upon phosphorylation by a kinase, a magnesium ion is chelated to form a bridge between the Sox moiety and the phosphate group. The presence of the magnesium chelate bridge results in an increase in fluorescence when the compound is excited at 360 nm and the emission measured at 485 nm.

Methods

Reaction Mix
5 µL of Syk kinase sample (0, 10, 20, 40 ng/well)
45 µL Master mix (proprietary buffer, 1 mM ATP, 1 mM DTT, 5 µM Peptide substrate)
Read kinetically every 30 seconds for 60 minutes at 30°C
Excitation: 360/40
Emission: 485/20
Dichroic: 400 nm cut off

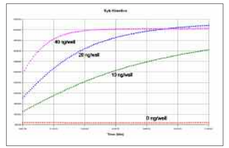

Figure 8. Fluorescence of Omnia® reactions with various concentrations of Syk kinase

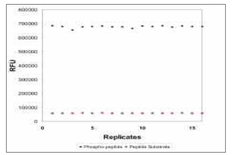

Figure 9. Statistical plot comparing phosphopeptide control to peptide substrate

Statistical Analysis	Result
Signal / Background ratio	11.3
Signal/Noise ratio	79.4
% CV	1.14
Z Value	0.96

Table 1. Statistical calculations based on the comparison between a phosphopeptide control and peptide substrate

Results

- Presence of Syk kinase resulted in an increase in fluorescence over time
- Rate of reaction was proportional to the amount of kinase
- Comparison between controls demonstrates significant statistical differences

LanthaScreen™ Assay

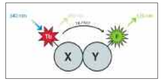

Figure 10. Principle of LanthaScreen™ TR-FRET detection.

Introduction

LanthaScreen™ technology uses time-resolved fluorescence resonance energy transfer (TR-FRET) to study multiple target classes including protein kinases, nuclear hormone receptors, as well as proteases and ubiquitinated proteins. LanthaScreen™ TR-FRET works on the principles that when suitable pairs of fluorophores are in close proximity of one another, excitation of the terbium chelate donor fluorophore results in energy transfer to the fluorescein or GFP acceptor fluorophore. The benefit of this assay is the long fluorescence lifetime of the terbium lanthanide donor fluor excited at 340 nm. This allows ratiometric measurement of acceptor fluorescence emission (FRET signal) at 520 nm over the 495 nm donor emission long after the background fluorescence has dissipated. The time-resolved measurement reduces assay interference (e.g. fluorescent compounds) and increases data quality.

Methods

Reaction
20 µL of Tb-anti-GST antibody (4 nM)
20 µL of Fluorescein-GST-MBP positive control dilution
Incubate for 60 minutes
Measure Fluorescence

Endpoint
Excitation: 340/30
Emission: 495/10 and 525/20
Dichroic: 400 nm cut off
Delay: 100 µsec
Collection Time: 200 µsec

Spectral Scans
Emission Scan: 400 nm to 700 nm
Increment: 1 nm
Excitation: 340 nm

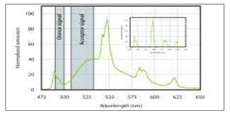

Figure 11. Fluorescence spectra of Terbium and Fluorescein (FRET) emission

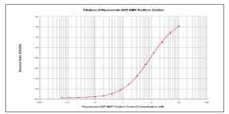

Figure 12. Titration of Fluorescein-GST-MBP with Tb-Anti-GST antibody in 384-well microplates

Results

- Spectral Scan demonstrates presence of Fluorescein FRET signal
- Increasing amounts of positive control result in an increase in the Emission Ratio

GeneBLAzer® Assay

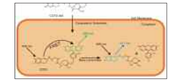

Figure 13. Schematic illustration of GeneBLAzer® Assay

Introduction

The basis for detection of the GeneBLAzer® assay is the membrane-permeant FRET based substrate. This noninvasive technique uses a CCF4 substrate where the carboxylate groups have been derivatized as esters resulting in a nonpolar compound (CCF4-AM) that is permeable to cell membranes. Once inside the cell, the compound is hydrolyzed by intracellular esterases. The resultant activated indicator is now a polar molecule that is no longer capable of freely diffusing through the cell membrane, essentially trapping the compound inside the cell. The CCF4 molecule has two fluorescent moieties, coumarin and fluorescein linked together with a beta-lactam ring. In the absence of beta-lactamase activity the unreacted substrate molecule remains intact. Excitation of the coumarin by 409 nm light results in fluorescence resonant energy transfer (FRET) to the fluorescein moiety, which can be detected by green 520 nm fluorescence. In the presence of beta-lactamase expression, the CCF4 substrate is cleaved at the beta-lactam ring and the FRET is disrupted. Under these conditions, excitation of the coumarin results in the emission of blue 447 nm fluorescence. Beta-lactamase expression is quantified by measuring the ratio of the blue product (447 nm) to the green substrate (520 nm) fluorescence.

Methods

Plate cells and allow overnight growth
Add inhibitor and agonist
Incubate 4-5 hours at 37°C with 5% CO_2
Load cells with LiveBLAzer™ substrate
Incubate for 2-2.5 hours
Measure Fluorescence with bottom optics
Excitation: 400/30
Emission: 460/40 and 528/20

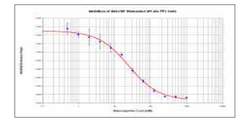

Figure 14. Staurosporine inhibition of β-lactamase expression in irf1-bla TF1 Cells cultured in 96-well microplates

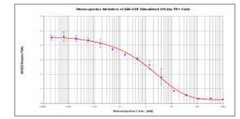

Figure 15. Staurosporine inhibition of beta-lactamase expression in irf1-bla TF1 cells cultured in 384-well microplates

Results

- The non-specific kinase inhibitor Staurosporine Inhibits β-lactamase expression in 96-well microplates
- This cell-based assay is easily transferred to the 384-well plate format.