The Synergy™ Family of Readers; A Lower Cost High Performance Alternative for Running AlphaScreen® Assays

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Abstract

AlphaScreen® assays are a homogeneous assay technology that is effective for screening a broad range of targets. This assay technology has been adapted to measure cAMP; Insulin; kinase activity; TNF-α; and IP_3 to name a few analytes. AlphaScreen® assays have historically been run using expensive detection systems that utilize a dedicated laser for excitatory light. Monochromatic lasers provide intense continuous light output as well as wavelength specificity, both of which are necessary for the AlphaScreen® assay. Xenon flash lamps, which are often used in multidetection microplate readers, provide light output over a broad wavelength range, but also cease illuminating very rapidly as required for time resolved measurements. Unfortunately, a xenon flash lamp does not provide the continuous illumination necessary for AlphaScreen® assays. The interval between flashes allows the excitatory reaction to diminish. Unlike most microplate readers, the BioTek Synergy™ 2 and Synergy™ 4 multi-mode readers incorporate both a constant light source as well as a xenon-flash lamp. Their unique multi-optic design provides continuous excitation, wavelength specificity, and high sensitivity necessary for these assays to be run effectively. Here we describe the data from several typical AlphaScreen® assays, including cAMP; insulin; TNF-α; as well as pJNK and p38MAPK phosphorylation, demonstrating the ability of the Synergy™ readers to perform this popular assay technology. Examples of typical performance of these assays will be provided along with an overview of both the assay technology and reader design.

AlphaScreen® Assays

Introduction

The Amplified Luminescent Proximity Homogeneous Assay (AlphaScreen®) uses active donor and acceptor beads that have been coated with a layer of hydrogel, which allows their conjugation with biological molecules (Figure 1). With excitation, a photosensitizer in the donor bead converts ambient oxygen to reactive singlet oxygen. A high concentration of the photosensitizer in the donor bead can result in the generation of as much as 60,000 singlet oxygen molecules per second and serves as a means for significant signal amplification. The singlet oxygen species reacts with thioxene compounds in the acceptor bead to generate a chemiluminescent signal that emits at 370 nm. The energy is immediately transferred to fluorophores contained in the same acceptor bead, which effectively shifts the emission wavelength to 520-620 nm. Because singlet oxygen is unstable, with an average lifetime of approximately 4 µsec, it can only diffuse a distance of 200 nm before it decays. The distance limitation insures that in the absence of a specific biological interaction between the two beads the singlet oxygen produced by the donor bead will go undetected. Acceptor beads that are not within this distance will not emit light.

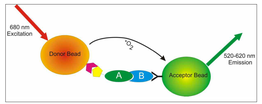

Figure 1. Schematic diagram of AlphaScreen® Reaction.

Figure 2. Synergy™ 4 Multi-Mode Microplate Reader with Hybrid Technology™.

The Synergy™ 2 and Synergy™ 4 are examples of a new type of reader that provides the combined benefit of bringing to research laboratories performances and technologies usually found on high-end HTS instrumentation, while at the same time delivering flexibility and efficient cost-control to screening laboratories (Figure 2). These readers utilize dedicated sets of optics to provide optimal performance regardless of the detection technology. Absorbance measurements use a xenon-flash lamp with a monochromator for wavelength selection, allowing the selection of any wavelength for endpoint or kinetic measures from 200 nm to 999 nm. On the Synergy™ 2 fluorescence measurements are made using bandpass filters with or without dichroic mirrors for wavelength selection and PMT for detection, while the Synergy™ 4 uses a unique Hybrid monochromator-filter system. 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™ readers integrate a high-energy xenon-flash-lamp with excitation and emission filters and PMT detector. Luminescence measurements are made using a liquid-filled optical fiber to capture light along with a low noise PMT. The Synergy™ readers are capable of reading plate formats up to 1536-wells, robotic compatible, provides temperature control, with shaking as standard features.

Here we describe the use of the Synergy™ family of readers to make AlphaScreen® determinations with a variety of different assay kit types.

Competitive AlphaScreen® Assay

Introduction

In the cAMP AlphaScreen® assay exogenously added biotinylated cAMP that is recognized by the anti-cAMP antibody conjugated to acceptor bead will compete with endogenous cAMP for binding. Only the biotinylated cAMP will be captured by both the donor bead's conjugated streptavidin and the cAMP specific antibody conjugated to the acceptor bead simultaneously resulting in an AlphaScreen® signal (Figure 3). Endogenous cAMP, which is recognized by the cAMP specific antibody, competes with and displaces biotinylated cAMP from antibody binding sites, but does not bind to the streptavidin conjugated to the donor bead. Increasing amounts of endogenous cAMP result in a decrease in signal.

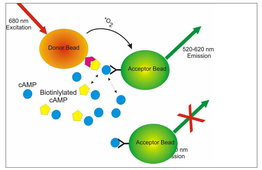

Figure 3. Schematic of competitive cAMP Assay.

Materials and Methods

An AlphaScreen® cAMP kit *(catalog #676062D)* was purchased from Perkin Elmer (Boston, MA).
Solid white 384-well microplates *(catalog #3705)* were obtained from Corning (Corning, NY).
PBS tablets *(catalog #P-4417)* were procured from Sigma-Aldrich (St. Louis, MO).
The AlphaScreen® cAMP kit was used as described by the kit instructions. Briefly, a series of dilutions of cAMP from 0 to 5×10^{-5} M were made from the 50 µM stock solution provided by the assay kit. Dilutions were made using 1X control buffer (5 mM HEPES, 50 mM NaCl, 0.03% Tween-20, pH 7.4) as the diluent. Control buffer was made daily by diluting a 10 X buffer and 3% Tween-20 stock solutions concurrently to 1X and 0.03% respectively with distilled water. In addition, several other working solutions were also prepared daily. A working solution of anti-cAMP Acceptor beads (1 Unit/5 µL) was made from the provided 10 Units/µL stock by dilution with 1X control buffer. Biotinylated cAMP provided in the assay kit was diluted to 3.3 Units/µL using 1X control buffer. Diluting the provided donor beads and the working biotinylated cAMP solution with 1X control buffer produced detection mix. To make 750 µL of detection mix, 5 µL of Donor beads (10 Unit/µL), 15 µL Biotinylated cAMP working solution (3.3 Units/µL) were mixed with 730 µL 1X control buffer, resulting in final concentrations for both of 1 Unit/15 µL. Assay reactions were aliquoted into solid white 384-well microplates in replicates of 8. To each well 5 µL of working anti-cAMP Acceptor bead solution was added followed by 5 µL of each cAMP dilution. The mixture was then incubated in the dark at room temperature for 30 minutes, after which 15 µL of detection mix was added to all wells. After incubation in the dark at room temperature for 60 minutes, the AlphaScreen® signal was determined on a Synergy™ 2 Multi-Mode Microplate Reader controlled by Gen5™ Data Analysis Software (BioTek Instruments).

Results

Figure 2 demonstrates the ability of the Synergy™ 2 to perform an AlphaScreen® assay to quantitate cAMP concentrations in unknown samples. When a range of cAMP concentrations are assayed according to the assay kit instruction a sigmoid shaped curve is observed if the AlphaScreen® signal is plotted as a function of cAMP concentration. Because the assay is a competitive assay, the signal decreases with increasing cAMP concentration. Using a 4-parameter logistical fit of the data a very reliable standard curve can be generated and unknown concentrations determined by interpolation.

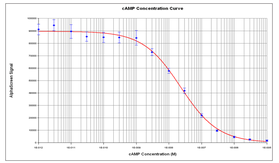

Figure 4. cAMP Concentration Curve.

Kinase Assays

Introduction

Cellular assays to detect the phosphorylation of endogenous proteins such as p38MAPK or pJNK can be carried out using AlphaScreen® technology. Both of these AlphaScreen® assays are *SureFire*™ kits where the donor and acceptor beads are common to many different assays, but specificity is provided by antibodies. These two assays use a biotin conjugated protein-specific antibody bound to a streptavidin coated donor bead in conjunction with an anti-phosphoprotein IgG antibody which is captured with a Protein A coated Acceptor bead. Only in the presence of phosphorylated product will both the donor and acceptor beads be in close enough proximity for AlphaScreen® reaction to take place. When cells are stimulated with different stimuli one can expect differential phosphorylation dependent on the stimuli. As observed in Figures 6 and 7 the Synergy™ 2 can detect the phosphorylation of JNK and p38MAPK proteins by various stimuli. In addition, the resolution is such that differential timing of increases in phosphorylation can be observed for these two proteins.

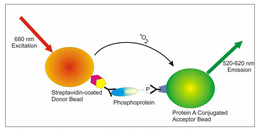

Figure 5. Schematic of SureFire™ Cellular Kinase Assay.

Materials and Methods

AlphaScreen® assays for p-p38MAPK and p-JNK proteins were carried out using *SureFire*™ assay kits. Reactions were carried according to the assay kit instructions. In each case cells were stimulated with EGF, Sorbitol, Anisomycin, and TNF-α and the degree of phosphorylation measured at 0, 5', 10', 15', and 30' after stimulation. Samples were placed in white 384-well plates and the AlphaScreen® signal measured using a Synergy™ 2 Multi-Mode Microplate Reader (BioTek Instruments).

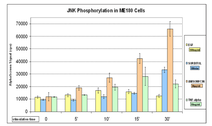

Figure 6. Phosphorylation of p-JNK protein after various stimuli.

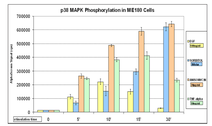

Figure 7. Phosphorylation of p-p38MAPK protein after various stimuli.

Results

Cellular assays to detect the phosphorylation of endogenous proteins such as p38MAPK or pJNK can be carried out using AlphaScreen® technology. Both of these AlphaScreen® assays are *SureFire*™ kits where the donor and acceptor beads are common to many different assays, but specificity is provided by antibodies. Only in the presence of phosphorylated product will energy transfer take place. When cells are stimulated with different stimuli one can expect differential phosphorylation dependent on the stimuli. As observed in Figures 6 and 7, the Synergy™ 2 can detect the phosphorylation of JNK and p38MAPK proteins by various stimuli. In addition, the resolution is such that differential timing of increases in phosphorylation can be observed for these two proteins.

Insulin AlphaLISA™ Assay

The human insulin assay uses two different monoclonal antibodies against insulin to bring the donor and acceptor beads in close proximity. The AlphaLISA™ acceptor beads have been conjugated with 8E2 anti-human insulin antibodies, which can bind and capture any human insulin in the sample. The biotinylated 3A6 anti-human monoclonal antibody forms a bridge with the streptavidin coated donor bead by binding the insulin-acceptor bead complex and the donor bead (Figure 8).

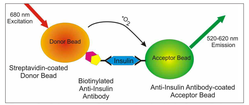

Figure 8. Schematic of Human Insulin AlphaScreen® Assay.

Materials and Methods

A Human Insulin Detection kit, which contained AlphaLISA™ acceptor beads, AlphaScreen® Donor-streptavidin beads, human Insulin, and biotinylated anti-human insulin antibody, was obtained as a generous gift from Perkin Elmer (Boston, MA). Solid white 384-well microplates *(catalog #3705)* were obtained from Corning (Corning, NY). A series of dilutions ranging from 0 to 400 µIU/mL of human insulin were made using assay buffer as the diluent. Assay buffer consisted of 30 mM Tris pH 7.4, 0.5% Triton X-100, 0.1% BSA. A master mix that contained biotinylated anti-human insulin-3A6 antibody (2.5 nM) and Anti-insulin 8E2 antibody coated Acceptor beads (25 µg/ml) was prepared using assay buffer as the diluent. Streptavidin coated donor beads were diluted 80-µg/mL using assay-buffer. Reactions were prepared by adding 5 µL of insulin dilution to wells of a 384-well microplate in replicates of 8 followed by the addition of 20 µL of master mix to all wells. This was allowed to incubate for 30 minutes at room temperature followed by the addition of 25 µL of diluted donor bead solution. The complete reaction mixture contained 1 nM biotinylated-3A6 antibody, 10 µg/mL acceptor beads and 40 µg/mL donor beads besides the human insulin dilution. The reaction was allowed to incubate in the dark for 60 minutes and the AlphaLISA™ signal determined using a Synergy™ 4 Multi-Mode Microplate Reader (BioTek Instruments). The reader was controlled and the data collected and analyzed using Gen5™ Data Analysis Software (BioTek Instruments).

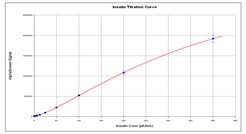

Figure 9. Human Insulin Concentration Curve. AlphaLISA™ signal generated from a titration of Human insulin was plotted against insulin concentration and a logistic 4-parameter best fit applied to the data using Gen5™ Data Analysis Software.

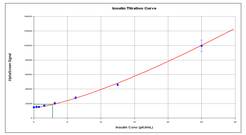

Figure 10. Titration of low Insulin Concentrations. The AlphaLISA™ signal and the concentration of the calculated detection limit are indicated by the green line.

Results

Figure 9 demonstrates the relationship between insulin concentration and AlphaLISA™ signal. This relationship can be best described using a 4-parameter logistic fit of the data. This assay directly quantifies insulin concentration and as such the AlphaLISA™ signal increases with increasing insulin concentrations. By using the "zoom" feature of the Gen5™ Data Analysis Software, one can examine the signal for low insulin concentrations. As seen in Figure 10, even at very low insulin concentrations the logistic fit of the data shows a very good relationship with the true data. The detection limit of the assay was determined from the calibration curve using the calculation of 3X the standard deviation above the zero standard. This value was then used to interpolate the calibration curve. An average value of 2.8 µIU/mL or 120 pg/mL was calculated as the limit of detection.

Conclusions

- The Synergy™ family of readers provide excellent results when measuring AlphaScreen® Assays.
- The Synergy™ readers are an economical alternative to dedicated laser based instrumentation.
- cAMP, Insulin, and Phospho-proteins are easily quantitated using Synergy readers.
- Wide variety of assays including AlphaLISA™, *SureFire*™ are available using AlphaScreen® technology.
- Gen5™ Software allows for both reader control and easy data analysis.