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Application Note

AlphaScreen™ Quantitation of cAMP using the Synergy™ 2 Multi-Mode Microplate Reader

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Adenosine 3', 5' cyclic monophosphate (cAMP) plays a critical role in the transmission of signals by functioning as a "second messenger". Binding of hormones or ligands to their respective receptors can either inhibit or enhance the production of cAMP by changing the activity of the enzyme adenylate cyclase. Adenylate cyclase is a membrane-associated enzyme that catalyzes the formation of cAMP from ATP. Intracellular cAMP levels, in turn, regulate enzymatic activity of numerous protein kinases, which phosphorylate specific targets setting off a cascade of cellular events. Because of the importance of cAMP as a cellular messenger, several different reading methodologies have been developed to measure cellular levels, one of which is AlphaScreen. Here we describe and demonstrate the ability of the Synergy™ 2 to quantitate cAMP using this methodology.

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.

The cAMP AlphaScreen assay is a competitive assay. Competitive assays generally have the analyte compete with exogenously added biotinylated analyte. For example, 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 1). 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.

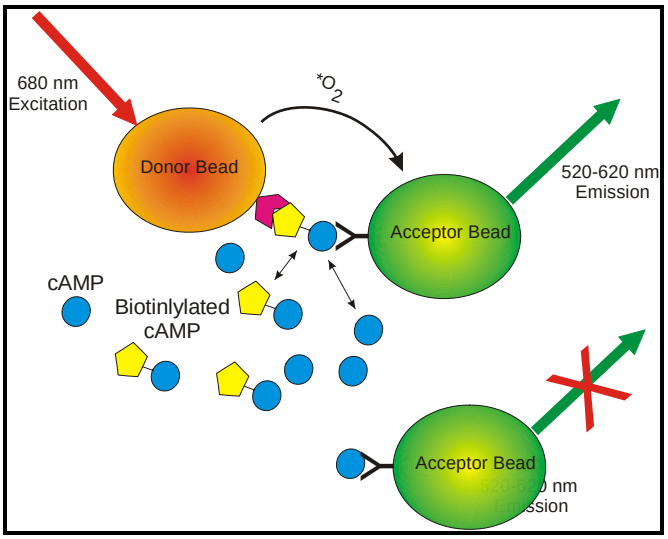

Figure 1. Schematic of competitive cAMP Assay.

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 x 10^{-5} M were made fromm 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 74.) 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 Units/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 reader 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.

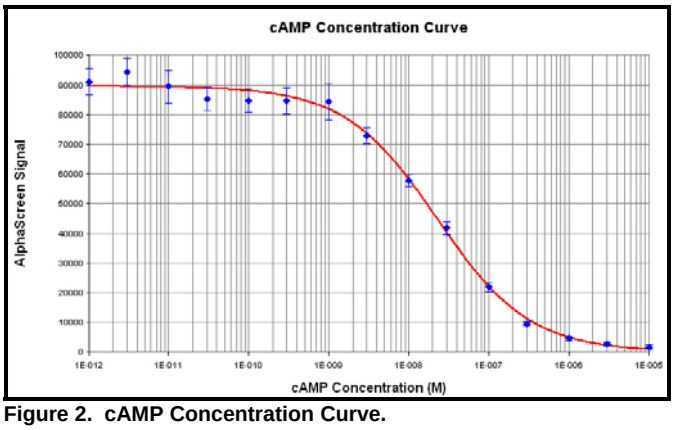

Figure 2. cAMP Concentration Curve.

Discussion

There are several aspects of the assay that should be considered when running the AlphaScreen cAMP assay. AlphaScreen beads are light sensitive. The cAMP assay should be performed under subdued light conditions. In this treatise, the assays were performed with the fluorescent light fixtures turned off using only indirect ambient light. The kit manufacturer (Perkin Elmer) recommends that lighting be under 100 Lux or that green filters be applied to lighting fixtures. Most importantly, any incubation should be performed in the dark or with plates covered with another plate of aluminum foil. The small volumes of the assay are prone to evaporation, as such the use of adhesive plate seals during incubations is recommended for optimal assay performance. Cellular assays that utilize cells grown in media containing high amounts of biotin (e.g. RPMI) can present a problem for the assay. Cells grown in such media should be rinsed with appropriate buffer such as PBS before they are used.

These data demonstrate the ability of the Synergy 2 Multi-Mode Microplate Reader to perform the AlphaScreen™ competitive cAMP assay. The Synergy 2 and Gen5 Data Analysis Software can make AlphaScreen determinations, perform any data transformation, and provide statistics and plot the data and calculate concentrations of unknown samples. The reader uses a continuous tungsten-halogen light source to excite the AlphaScreen and in conjunction with a unique two filter pair read technique provides very good results. The competitive assay shows a large signal-change, taking place over 4-5 decades of analyte concentrations. With low sample replicate variations analysis of samples can be made with a high degree of confidence.

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