

AlphaScreen cGMP Phosphodiesterase Assay Using AlphaScreen Biotinylated cGMP Supplement (Cat No. 6760306M/R)

Introduction

Guanosine 3',5'-cyclic monophosphate (cGMP) is an ubiquitous intracellular second messenger that acts as a regulator of vascular smooth muscle cell contractility, growth and differentiation. cGMP is formed either by membrane-bound guanylate cyclase activated by natriuretic peptides or by soluble guanylate cyclases activated by nitric oxide (NO). On the other hand, phosphodiesterases (PDE) catalyze the breakdown of cGMP to its corresponding 5' nucleotide. PDEs represent important therapeutic targets and, over the last several years, many novel agents have been identified that exert selective inhibitory effects on the various molecular forms of PDE (Ex: Viagra®, an inhibitor of PDE Type V). There is a growing interest to develop selective PDE inhibitors.

We present here the development of a cGMP-specific PDE Type V assay using the AlphaScreen™ Biotinylated cGMP Supplement. This supplement was designed for the measurement of both guanylate cyclase and cGMP-specific phosphodiesterase activities using the AlphaScreen technology. In this application

note, we present the development of a cGMP-specific PDE assay. The results on the cGMP guanylate cyclase assay were previously published in a poster entitled: '*Development of a non-radioactive homogeneous HTS platform to measure the activity of guanylate cyclase*' (Presented at the SBS 2004 and available from the PerkinElmer website).

Principles of AlphaScreen technology

AlphaScreen is a bead-based non-radioactive Amplified Luminescent Proximity Homogeneous Assay. When a biological interaction brings the beads together, a cascade of chemical reactions act to produce a greatly amplified signal. On laser excitation, a photosensitizer in the Donor bead converts ambient oxygen to a more excited singlet state. The singlet state oxygen molecules diffuse across to react with a thioxene derivative in the Acceptor bead, generating chemiluminescence at 370 nm that further activates fluorophores contained in the same bead. The fluorophores subsequently emit light at 520-620 nm. In the absence of a specific biological interaction,

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the singlet state oxygen molecules produced by the Donor bead go undetected without the close proximity of the Acceptor bead. As a result only a very low background signal is produced. AlphaScreen provides a highly versatile, sensitive, time-resolved, homogeneous and miniaturizable means to efficiently perform assay development and HTS resulting in higher throughput at lower costs. To maximize AlphaScreen signal detection, the AlphaQuest®-HTS, the Fusion-Alpha™ and EnVision™ Microplate Analyzers were developed with the capability to measure assays in multi-well plates. These instruments use a highly efficient laser diode emitting at 680 nm, fiber optics and specially optimized photomultiplier tubes. For further details on AlphaScreen technology, refer to 'A Practical Guide to Working with AlphaScreen' (PerkinElmer reference no. S4077).

AlphaScreen cGMP phosphodiesterase assay

The AlphaScreen cGMP phosphodiesterase assay aims at measuring the activity of cGMP-specific PDE enzymes. The assay is based on the binding of the AlphaScreen Biotinylated cGMP Supplement (biotinylated cGMP tracer) to a specific anti-cGMP antibody. The AlphaScreen Biotinylated cGMP Supplement and the antibody are respectively captured by the AlphaScreen streptavidin-Donor and Protein A conjugated Acceptor beads. The high-affinity interaction between the AlphaScreen cGMP supplement and the antibody allows the two types of beads to come into close proximity, resulting in an intense AlphaScreen signal. Unlabelled cGMP competes against the biotinylated cGMP tracer on the antibody binding sites resulting in a concentration-dependent signal

decrease. cGMP-specific phosphodiesterases (PDEs) are enzymes catalyzing the conversion of unlabelled cGMP into the 5' GMP nucleotide. As GMP is not recognized by the anti-cGMP antibody, an increase in the AlphaScreen signal is observed as a result of the PDE activity.

Materials and methods

Materials

The AlphaScreen cGMP PDE assay uses the AlphaScreen Biotinylated cGMP Supplement (cat No. 6760306M or 6760306R) which is a biotinylated cGMP derivative. This assay also requires the AlphaScreen Protein A detection kit (PerkinElmer cat. No. 6760617) composed of Protein A acceptor beads and Streptavidin donor beads, and a specific anti-cGMP antibody. For this study, rabbit poly-clonal anti-cGMP antibodies from BioVision (cat. No. 3568-100) and from Chemicon (cat. No. AB303) were used. The recombinant PDE Type V was purchased from Calbiochem (cat. No. 524715). cGMP was obtained from Sigma (cat. No. G6129).

Buffers

- Assay buffer: 25 mM Hepes (pH 7.4), 100 mM NaCl, 0.1% Tween-20.
- PDE reaction buffer: 25 mM Hepes (pH 7.4), 2.5 mM MgCl₂ and 0.1% BSA.
- Stop/Detection buffer: 25 mM Hepes (pH 7.4), 100 mM NaCl, 0.1% Tween-20, 25 mM EDTA.

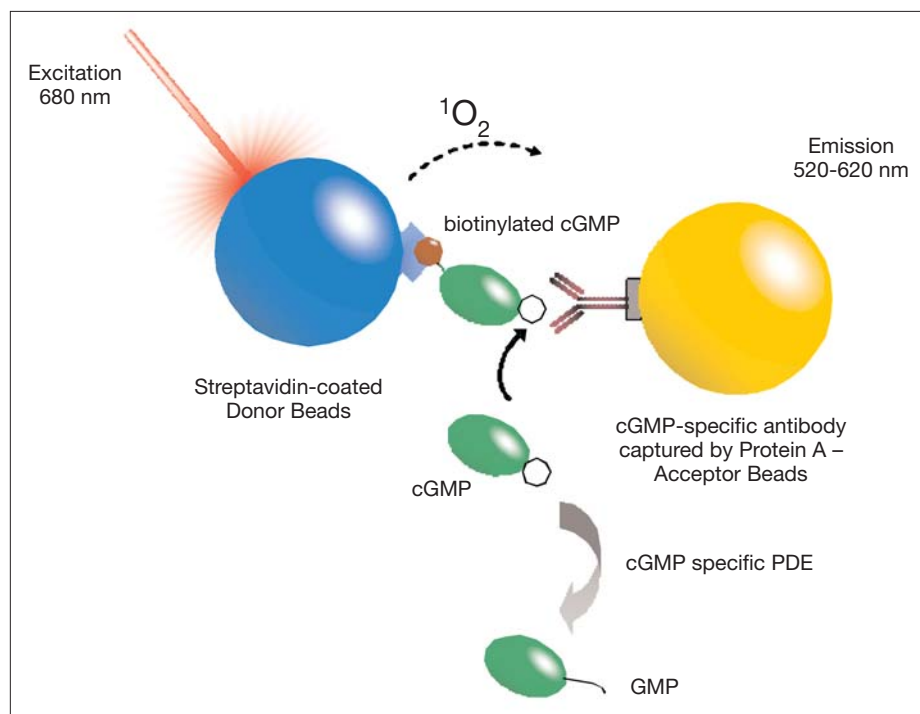


Figure 1. Principle of the AlphaScreen cGMP PDE assay. The cGMP PDE enzyme depletes the unlabelled cGMP competitor, allowing the two AlphaScreen beads to bind via the anti-cGMP antibody interaction. As a result, an intense AlphaScreen signal is generated.

Protocol for generating a cGMP Standard Curve

The 384 assays were performed in white, opaque 384-well plates (PerkinElmer® Optiplate 384-well plate cat. No. 6007290 and 6007299) in a final volume of 25 µL. The 1536 assays were performed in white, opaque 1536-well plates (PerkinElmer Optiplate 1536-well plate cat. No. 6005228) in a final volume of 7.5 µL.

The AlphaScreen cGMP Supplement stock at 1.25 µM was diluted to 2.5 nM in Assay buffer. Serial dilutions of cGMP were prepared in Assay buffer (0-1 µM). The detection mix was prepared by diluting the AlphaScreen streptavidin-Donor beads and Protein A-Acceptor beads to 50 µg/mL, along with the anti-cGMP antibody at the appropriate dilution in the Assay buffer. The antibody from BioVision was diluted to 1:2,000 (1:5,000 final) and the antibody from Chemicon to 1:20,000 (1:50,000 final). The detection mix was allowed to incubate for 30 minutes at room temperature.

The cGMP standard curve assay in the 384 format involves the following steps:

1. Add 5 µL of cGMP dilutions (0-1 µM final conc.) to the plate
2. Add 10 µL of the AlphaScreen cGMP Supplement dilution (1 nM final)
3. Add 10 µL of Detection mix (anti-cGMP antibody / streptavidin-Donor beads (20 mg/mL final) / Protein A-Acceptor beads (20 µg/mL final))
4. Incubate the plate for 1 hour at room temperature in the dark and detect the AlphaScreen signal using either an AlphaQuest-HTS or a Fusion-α or an EnVision microplate analyzer.

The protocol for the 1536 assay is as follows:

1. Add 1.5 µL of cGMP dilutions (0-1 µM final conc.) to the plate
2. Add 3 µL of the AlphaScreen cGMP Supplement dilution (1 nM final)
3. Add 3 µL of Detection mix (anti-cGMP antibody / streptavidin-Donor beads (20 µg/mL final) / Protein A-Acceptor beads (20 µg/mL final))
4. Incubate the plate for 1 hour at room temperature in the dark and detect the AlphaScreen signal using either an AlphaQuest-HTS or a Fusion-α or an EnVision microplate analyzer.

Protocol for the cGMP PDE assay

The inhibitors tested in this study were diluted in the PDE reaction buffer supplemented with 400 nM cGMP. The PDE Type V stock solution was diluted to 1 U/µL in the PDE reaction buffer (not supplemented). The AlphaScreen cGMP Supplement stock solution at 1.25 µM was diluted to 2.5 nM in the Stop/Detection buffer. The detection mix was prepared by diluting the AlphaScreen streptavidin-Donor beads and Protein A-Acceptor beads to 50 µg/mL, and the anti-cGMP antibody at the appropriate dilution in the Assay buffer. The antibody from BioVision is diluted to 1:2,000 (1:5,000 final) and the antibody from Chemicon to 1:20,000 (1:50,000 final). The detection mix was allowed to preincubate for 30 minutes at room temperature.

The cGMP PDE assay involves the following steps:

1. Add 5 µL of inhibitor*
2. Add 5 µL of PDE Type V (5 U per well)

3. Incubate 120 minutes at 23 °C for enzymatic cleavage of unlabelled cGMP
4. Add 5 µL of the AlphaScreen cGMP Supplement (1 nM final)
5. Add 10 µL Detection mix (anti-cGMP antibody / streptavidin-Donor beads (20 µg/mL final) / Protein A-Acceptor beads (20 µg/mL final))
6. Incubate the plate for 1 hour at room temperature in the dark and detect the AlphaScreen signal using either an AlphaQuest-HTS or a Fusion-α or an EnVision microplate analyzer.

*Please note that for compound screening purposes, we recommend adding 0.5 µL of test compounds to 4.5 µL of cGMP substrate solution prepared in the PDE reaction Buffer. If compounds are diluted in 100% DMSO, this will bring the final DMSO concentration down to 2% in the assay which is a safe concentration (see below).

Results

A highly sensitive AlphaScreen cGMP assay was developed using the AlphaScreen Biotinylated cGMP Supplement (biotinylated cGMP tracer), the AlphaScreen Protein A kit and rabbit polyclonal anti-cGMP antibodies. Two different sources of antibody were tested: the antibody from BioVision (cat. No. 3568-100) and from Chemicon (cat. No. AB303) which were used at final dilutions of 1:5,000 and 1:50,000, respectively. When working with other sources of antibody, it is strongly recommended to ensure that the antibody is recognized by Protein A, and to determine the optimal concentration of antibody to use in the assay by performing an antibody titration assay before optimizing the PDE assay.

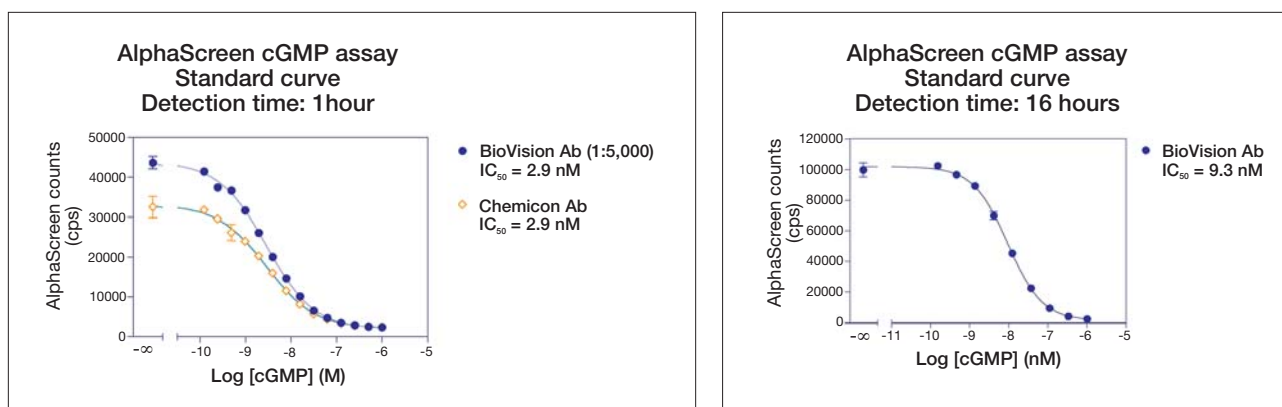


Figure 2. cGMP standard curves generated in 384-well plates (25 μ L) with the anti-cGMP antibodies obtained from BioVision and Chemicon. Left panel: 1 hour of detection time; right panel: 16 hours of detection time.

Figure 2 presents cGMP standard curves generated with both BioVision and Chemicon antibodies in 384-well microplates using a final assay volume of 25 μ L and a detection time of 1 hour (for both antibodies) or 16 hours (for BioVision antibody only).

As expected for a competition assay, increasing concentrations of cGMP led to a concentration-dependent decrease of the signal (Figure 2). After one hour incubation, both antibodies produced a total signal higher than 30,000 cps with signal-to-background (S/B) ratios of 20 (left panel). In both cases, the cGMP competitor efficiently displaced the binding of the AlphaScreen Biotinylated cGMP Supplement to the antibody binding sites with an IC₅₀ value of 2.9 nM, corresponding to 72 femtomoles of cGMP per well. The detection limit of the assay was determined to be 10-20 femtomoles of cGMP. As a result of a longer incubation period (16 hours, see right panel), the S/B ratio improved and the assay sensitivity was not significantly decreased, indicating that the assay can easily be read after an overnight incubation.

The assay was miniaturized in the 1536 format (total assay volume of 7.5 μ L) by decreasing the reagent volumes added to the plate and

keeping constant the reagent concentration. A typical cGMP standard curve generated with the BioVision antibody is presented in Figure 3. Excellent assay performance parameters were obtained, as demonstrated by an IC₅₀ value of 1-2 nM and a S/B ratio of 16.

Taken together, these 384 and 1536 data demonstrate the ability of the AlphaScreen cGMP assay at detecting very low amounts of cGMP in both 384 and 1536 formats.

Optimization of the AlphaScreen PDE assay using the PDE Type V enzyme

The AlphaScreen cGMP PDE assay was developed with a purified recombinant PDE Type V enzyme,

purchased from Calbiochem. The anti-cGMP antibody from BioVision was used in all subsequent experiments at a final dilution of 1:5,000. The optimization of the assay involves several steps which are:

Determination of the optimal concentrations of enzyme and substrate

Since the assay is based on the competition of unlabelled cGMP substrate and its reversal by the PDE activity, the first optimization step should be to titrate cGMP and the PDE enzyme. Minimal amounts of these two reagents should be determined to produce an assay with high sensitivity and a good window, while maintaining low cost benefits.

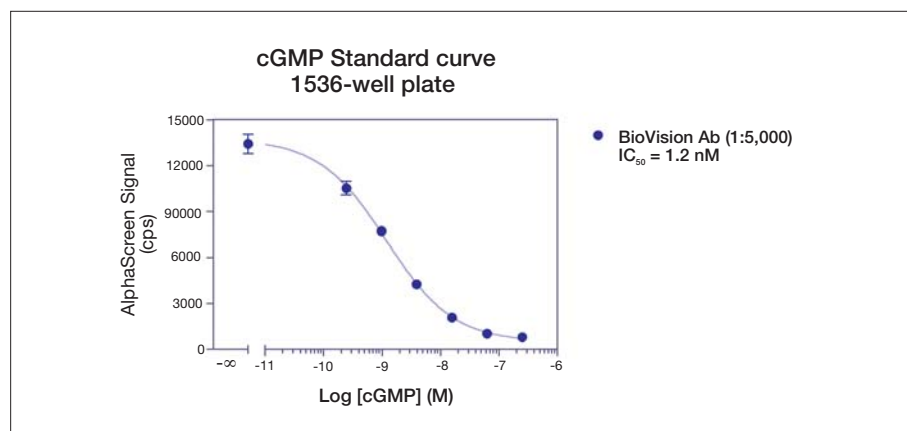


Figure 3. cGMP standard curves generated in 1536-well plates (7.5 μ L) with the anti-cGMP antibody obtained from BioVision.

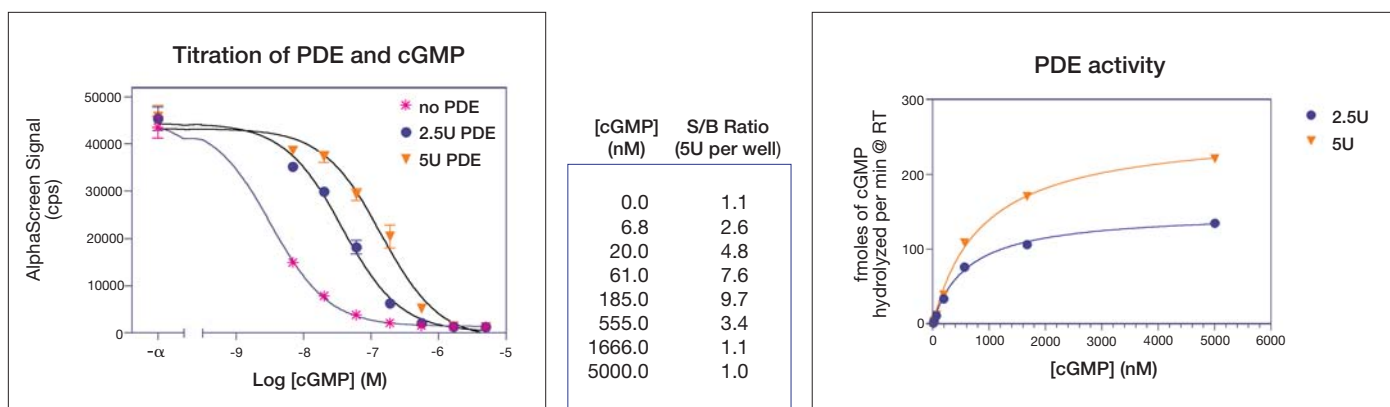


Figure 4. Titration of enzyme and substrate in the PDE assay. A detection time of 2 hours was used.

As observed in Figure 4, the optimal response, defined as the optimum S/B ratio measured between the basal signal (obtained in the absence of PDE) and the maximal enzymatic activity (obtained in the presence of PDE), was achieved when using 200 nM of cGMP and 5 units of PDE Type V. Under these conditions, a S/B ratio of approximately 10 was observed. These conditions were used in subsequent experiments.

Time Course of enzymatic activity

The second optimization step consists in conducting a time-course experiment at room temperature (23 °C) to optimize the reaction time for the PDE assay. After

incubation at different times, the enzymatic reaction was efficiently stopped by the addition of EDTA present in the stop/detection buffer.

As seen in Figure 5 Left Panel, the AlphaScreen signal increased as a function of time with a response nearly linear up to 150 minutes. The quantity of cGMP hydrolyzed at each time point was calculated from a cGMP standard curve (Right Panel). Since the signal was inclined to plateau at higher incubation times, we selected 120 min as the optimal incubation time for the PDE enzymatic reaction at 23 °C. At this time point, the amount of cGMP hydrolyzed was maximal (Right Panel). All subsequent experiments were performed using this time.

Signal stability

The stability of the signal is an important parameter in HTS applications. For that reason, we tested the stability of the AlphaScreen signal in the PDE assay following the addition of the stop/detection mix (anti-cGMP antibody / streptavidin-Donor / Protein A-Acceptor beads). Figure 6 presents the signals obtained in the presence or absence of PDE (total and non specific signals) using a detection time of 1-2-3-4-5 or 16 hours.

A proportional increase in total and non-specific signals was observed as a function of time, with no change in the S/B ratio up to 5 hours. Upon longer incubation

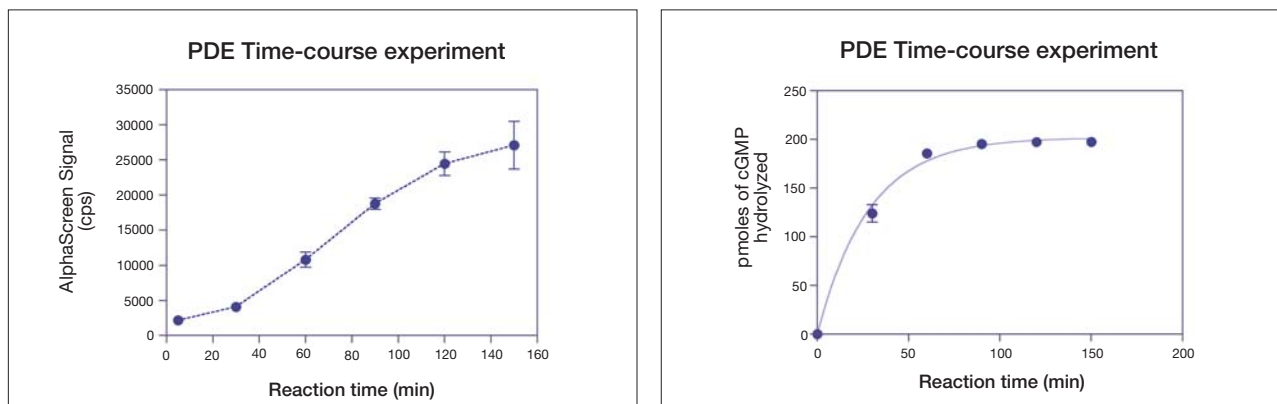


Figure 5. Time Course of PDE enzymatic activity. Left Panel: AlphaScreen Signal (cps); Right Panel: amount of cGMP hydrolyzed (pmoles).

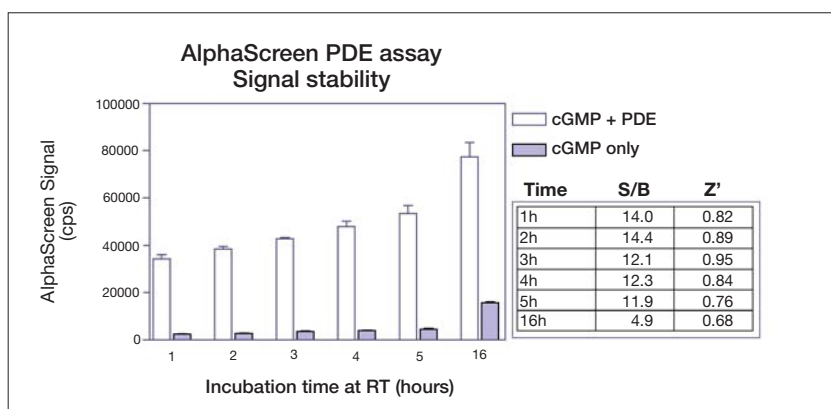


Figure 6. Signal stability over time.

periods (16 hours), the S/B ratio was shown to reduce as a result of an increased non-specific signal. However, the robustness of the assay as described by the Z' value was not significantly affected at this time point, demonstrating that the assay tolerates reading the plates following an overnight incubation. This allows screeners to perform both on-line and off-line reading.

Inhibition of the PDE activity

To verify the specificity of the enzymatic activity as well as the enzyme inhibitory profile, two well-described cGMP-specific PDE inhibitors were tested on the PDE Type V: (1) zaprinast, which is a generic cGMP PDE inhibitor, and (2) the compound

4-[[3',4'-(Methylenedioxy)benzyl]amino]-6-methoxyquinazoline, which is known to be a specific inhibitor of PDEs Type V. The assay was performed using a detection time of 1 and 16 hours.

As shown in Figure 7, the two compounds inhibited the PDE V enzyme with IC_{50} values in the nanomolar range (100-300 nM). Those values are in agreement with those reported in the literature (130 nM and 230 nM for Zaprinast and the Calbiochem's inhibitors respectively, See Ref. Takase *et al.*, 1994; Turko *et al.*, 1999). No significant change in the IC_{50} value was observed after an overnight incubation, confirming the stability of the assay.

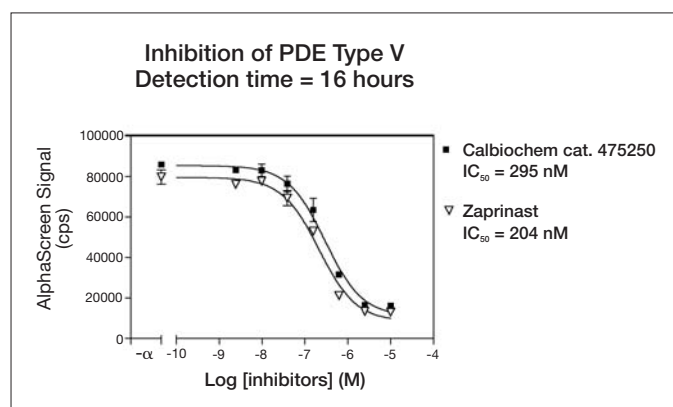
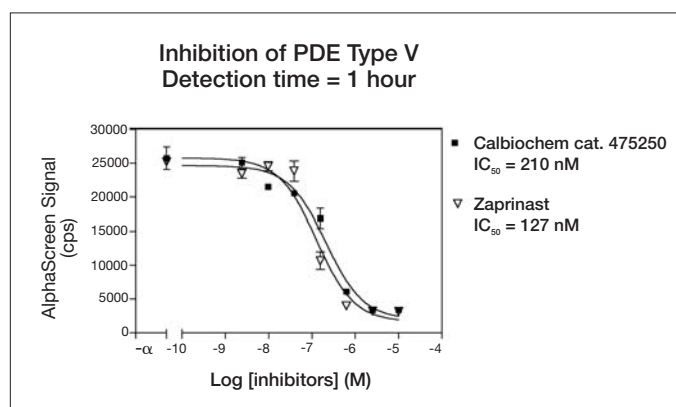


Figure 7. Inhibition Curves for PDE Type V.

DMSO Tolerance

In order to verify that the assay is amenable to HTS, the AlphaScreen cGMP PDE assay was tested for its sensitivity to increasing DMSO concentrations (see Figure 8).

The assay was shown to tolerate at least 6% DMSO in the enzymatic reaction without any loss of signal. This tolerance is compatible with the addition of 0.5 μ L of test compounds diluted in 100% in a 25 μ L assay. As expected, the signal was affected by higher DMSO concentrations, as shown by a 30% and 80% reduction in total signal in the presence of 13% and 25% DMSO, respectively.

Screening validation

While there are many parameters to assess assay quality, the Z-factor is the most popular among assay developers. Typically, a Z value greater than 0.5 is considered good, while assays with Z values greater than 0.7 are excellent (Zhang *et al.*, 1999). A Z' study was manually conducted on the cGMP PDE assay to assess the robustness of the cGMP PDE assay in the 384 format. Two populations of 48 replicates were generated in the presence or absence of 10 μ M Zaprinast inhibitor diluted in the PDE

Reaction Buffer containing 5% DMSO (final concentration in 10 μ L enzymatic reaction). Figure 9 presents typical results obtained after 1 hour of detection time. A Z' value of 0.81 and a S/B ratio of 13.7 were calculated from the two populations of data obtained with manual dispensing. These data strongly suggest that the assay is robust and suitable for compound screening and HTS campaigns.

Conclusion

The AlphaScreen cGMP detection assay which uses the AlphaScreen Biotinylated cGMP Supplement is very sensitive, allowing the detection of 10-20 fmoles of cGMP per well in 384-well and 3-6 fmoles of cGMP in 1536-well format. This assay is perfectly-suited for the measurement of guanylate cyclase or phosphodiesterase activities. We validated the performance of the AlphaScreen cGMP detection supplement by performing PDE assay using a recombinant cGMP-specific PDE Type V. The optimized assay was shown to be robust and specific, combining a high level of sensitivity with cost-effectiveness properties. The data presented here consolidate the idea that the AlphaScreen technology can be used as a plug-n-play detection platform for probing multiple molecular interactions with an outstanding readout quality.

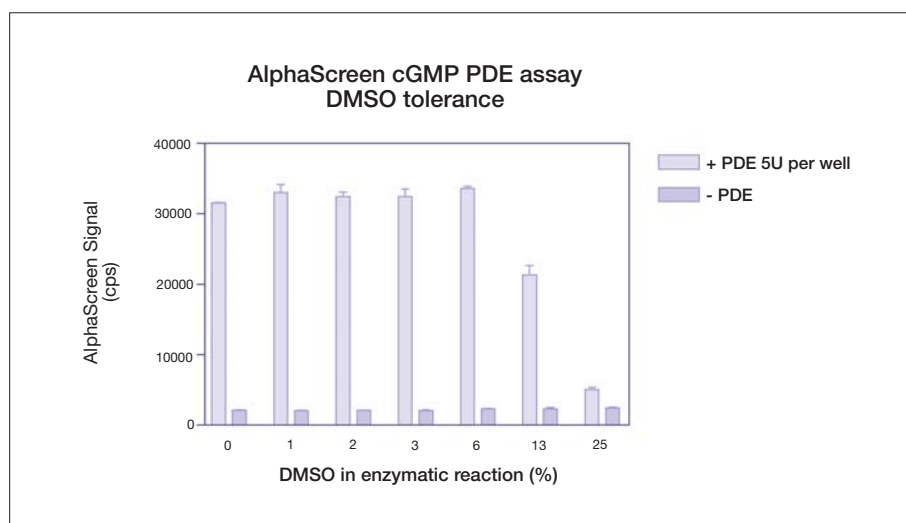


Figure 8. DMSO Tolerance of the PDE cGMP assay.

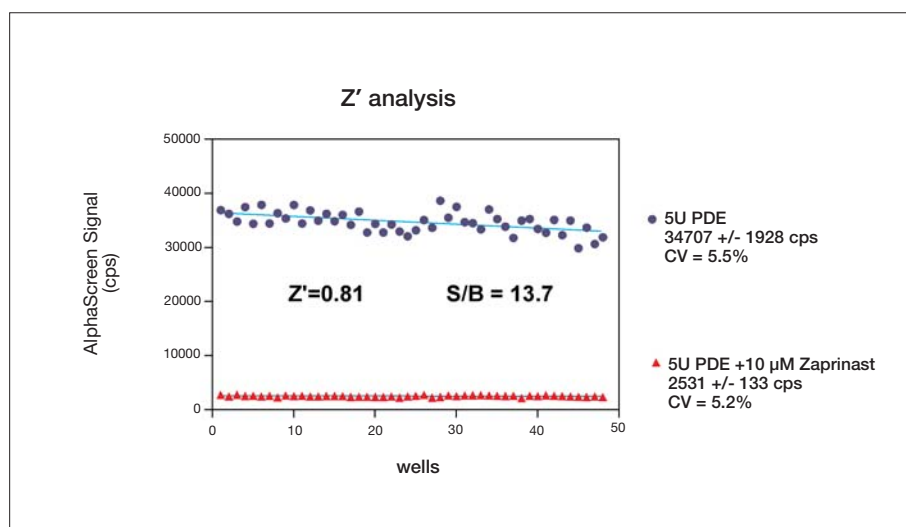


Figure 9. Screening validation of AlphaScreen cGMP PDE assay. Representative Z' graph for a 384 manual assay.

References

- Takase Y., Saeki T, Watanabe N, Adachi H, Souda S, Saito I. *J Med Chem.* 1994 Jun 24;37(13):2106-11
- Turko IV, Ballard SA, Francis SH, Corbin JD. *Mol Pharmacol.* 1999 Jul;56(1):124-30.
- Rouleau N., Boissonneault M., Roby P., Hudon-David F., Lasalle T., Bossé R. Development of a non-radioactive homogeneous HTS platform to measure the activity of guanylate cyclase.
- Zhang, J. *et al.* (1999) *J. Biomol. Screen.* 4, 67-73.

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