

AlphaScreen cGMP Phosphodiesterase Assay Using AlphaScreen cGMP Supplement with Anti-cGMP Antibody

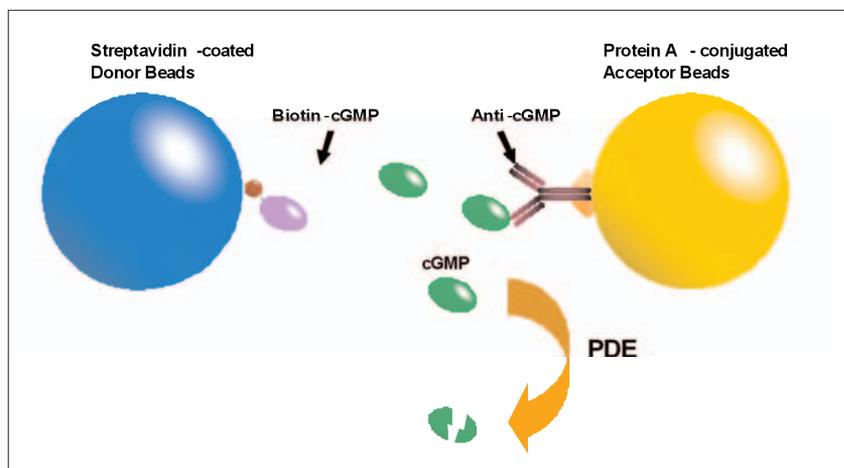


Figure 1. Principle of the AlphaScreen cGMP PDE assay. The cGMP PDE enzyme depletes the unlabeled cGMP competitor, allowing the two AlphaScreen beads to bind via the anti-cGMP antibody interaction. As a result, an intense AlphaScreen signal is generated.

Introduction

Guanosine 3',5'-cyclic monophosphate (cGMP) is a ubiquitous intracellular second messenger that acts as a regulator of vascular smooth muscle cell contractility, growth and differentiation. Two forms of guanylate cyclases are known to produce cGMP. The first form is called particulate¹ and is associated with the atrial natriuretic peptide (ANP) receptor function. The second form is of cytosolic nature². Phosphodiesterases (PDE's) catalyze the breakdown of cGMP to its corresponding 5' nucleotide derivative.

PDE's represent important therapeutic targets. Over the last years, several new drugs that exert selective inhibitory effects on the various molecular forms of PDE have been identified (Ex: Viagra[®], an inhibitor of PDE V). There is a growing interest to develop more specific PDE inhibitors.

In this application note, we present the development of an AlphaScreen[®] assay to measure the activity of the cGMP-specific PDE V assay using the AlphaScreen cGMP supplement with antibody product (cat. No. 6360308). This supplement was specifically designed to measure both guanylate cyclase and cGMP-specific phosphodiesterase activities.

The assay is based on the binding of the AlphaScreen cGMP supplement (biotinylated cGMP tracer) to a specific anti-cGMP antibody. The AlphaScreen cGMP supplement and the antibody are respectively captured by the AlphaScreen Streptavidin-conjugated Donor and Protein A-conjugated Acceptor beads (Figure 1). The high-affinity interaction between the AlphaScreen cGMP supplement and the antibody draws the two types of beads into close

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proximity, leading to the production of an intense AlphaScreen signal. Endogenous unlabeled cGMP competes against the biotinylated cGMP tracer on the antibody binding sites resulting in a concentration-dependent signal decrease. As the 5-cGMP produced by PDE V is not recognized by the anti-cGMP antibody, an increase in the AlphaScreen signal is observed as a result of the PDE V activity.

Materials and methods

Materials

The AlphaScreen cGMP PDE assay uses PerkinElmer's AlphaScreen cGMP Supplement with antibody (cat. no. 6760308M or 6760308R) which consists of a biotinylated cGMP derivative and an anti-cGMP antibody. The assay also requires the AlphaScreen Protein A detection kit (PerkinElmer cat. no. 6760617), composed of Protein A acceptor beads and Streptavidin donor beads. The recombinant PDE V and the -[3',4'-(Methylenedioxy)benzyl]amino]-6-methoxyquinazoline were purchased from Calbiochem® (cat. no.524715 and 475250, respectively). cGMP and Zaprinast were obtained from Sigma-Aldrich™ (cat no.G6129 and Z0878, respectively).

Method

To perform the cGMP PDE assay, inhibitors tested were diluted in the PDE reaction buffer supplemented with 400 nM cGMP. The PDE V stock solution was diluted to 1 unit / μ L in the PDE reaction buffer (25 mM Hepes pH 7.4, 2.5 mM MgCl₂, 0.1% BSA). The 1.25 μ M AlphaScreen cGMP Supplement stock solution was diluted to 2.5 nM in the Stop/ Detection buffer (25 mM Hepes pH 7.4, 100 mM NaCl, 0.1% Tween-20, 25 mM EDTA). The detection mix was prepared by diluting the AlphaScreen Streptavidin-Donor beads and Protein A-Acceptor beads to 50 μ g/mL, and the anti-cGMP antibody (1:2000) in the Assay buffer. The detection mix was allowed to pre-incubate for 30 minutes at room temperature.

The cGMP PDE assay was performed following these steps:

- 1- Add 5 μ L of inhibitor*
- 2- Add 5 μ L of PDE V (5U per well)
- 3- Incubate 120 minutes at 23 °C for enzymatic cleavage of unlabeled cGMP
- 4- Add 5 μ L of the AlphaScreen cGMP Supplement (1 nM final)

- 5- Add 10 μ L Detection mix (anti-cGMP antibody / Streptavidin-Donor beads (20 μ g/mL final) / Protein A-Acceptor beads (20 μ g/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 μ L of test compounds to 4.5 μ L of cGMP substrate solution prepared in the PDE reaction Buffer. If the test compounds are diluted in 100% DMSO, this will bring DMSO to a safe 2% final concentration in the assay.

Results and discussion

The sensitivity of the cGMP supplement with antibody product was initially characterized using exogenous cGMP in a 384-well microplate in a final assay volume of 25 μ L. Based on the cGMP standard curves generated, the detection limit of the assay is around 10-20 femtomoles of cGMP per well. Signal-to-background ratios of more than 20 were obtained, with an IC₅₀ of 2.9 nM and the signal was stable more than 16 hours, allowing for overnight incubation and off-line readings (data not shown).

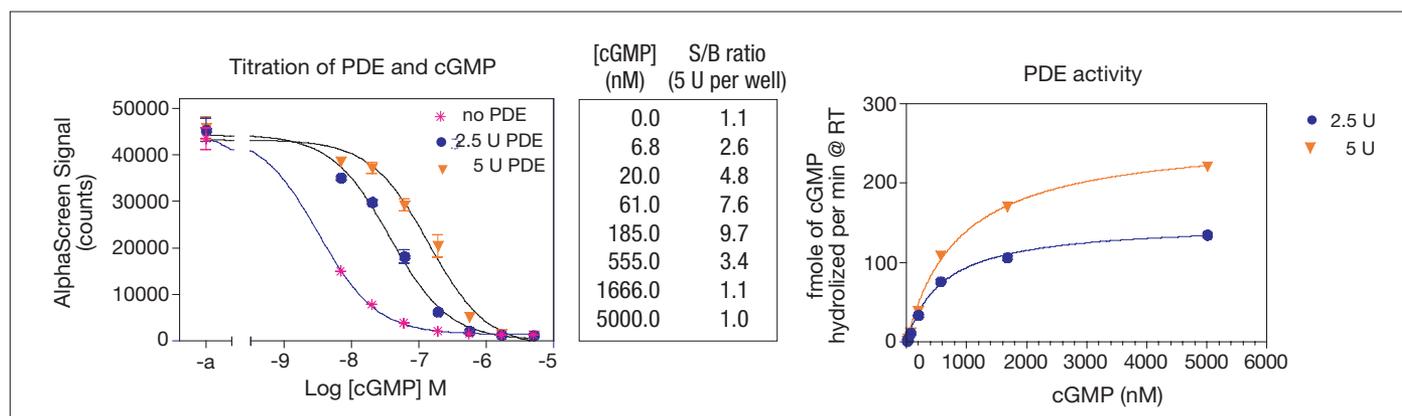


Figure 2. Titration of enzyme and substrate in the PDE assay using a detection time of 2 hours.

Optimization of the AlphaScreen PDE assay using recombinant PDE V

1) Determination of the optimal concentrations of enzyme and substrate

The first optimization step was to titrate cGMP and the PDE enzyme. The optimal response was defined as the highest S/B ratio measured between the basal signal obtained in the absence of PDE and the maximal signal (maximal enzymatic activity) recorded in the presence of PDE. As shown in Figure 2, an optimum S/B ratio of 10 was achieved using 200 nM of cGMP and 5 units of PDE V. These conditions were used in subsequent experiments. These parameters compare advantageously to other available technologies, allowing one to perform cost effective screens.

2) Time Course of Enzymatic Activity

To further optimize assay conditions, a detailed time-course analysis of PDE V activity was carried out at room temperature (23 °C). After incubation, the enzymatic reaction was efficiently stopped by the EDTA present in the stop/detection buffer. As seen in Figure 3 (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 at 23 °C (Figure 3, right panel). Since the signal plateaued at prolonged incubation time intervals, we selected 120 minutes as the optimal incubation time for all subsequent PDE enzymatic reactions.

3) Inhibition of the PDE activity

The specificity of the PDE V enzymatic activity as well as the enzyme inhibitory profile were assessed using two well-described cGMP-specific PDE inhibitors: the generic inhibitor Zaprinast and 4-[[3',4'-(Methylenedioxy)benzyl]amino]-6-methoxyquinazoline (4MBA), a PDE V inhibitor. The assay was performed using detection times of 1 and 16 hours to demonstrate the assay's stability with time. As shown in Figure 4, the two compounds inhibited the PDE V enzyme with IC_{50} values in the nanomolar range (100-300 nM), values that are in agreement with those reported in the literature (130 nM and 230 nM for Zaprinast and 4MBA, respectively)^{4, 5}. No significant changes in the IC_{50} values were observed after an overnight incubation, confirming the stability of the assay.

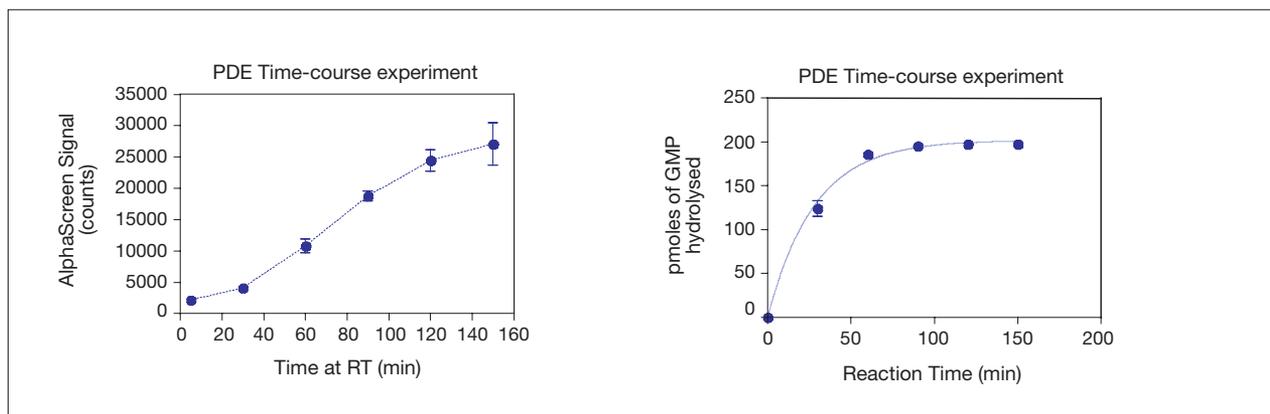


Figure 3. Time Course of PDE enzymatic activity. Left panel: AlphaScreen Signal (cps); Right Panel: amount of cGMP hydrolyzed (pmoles)

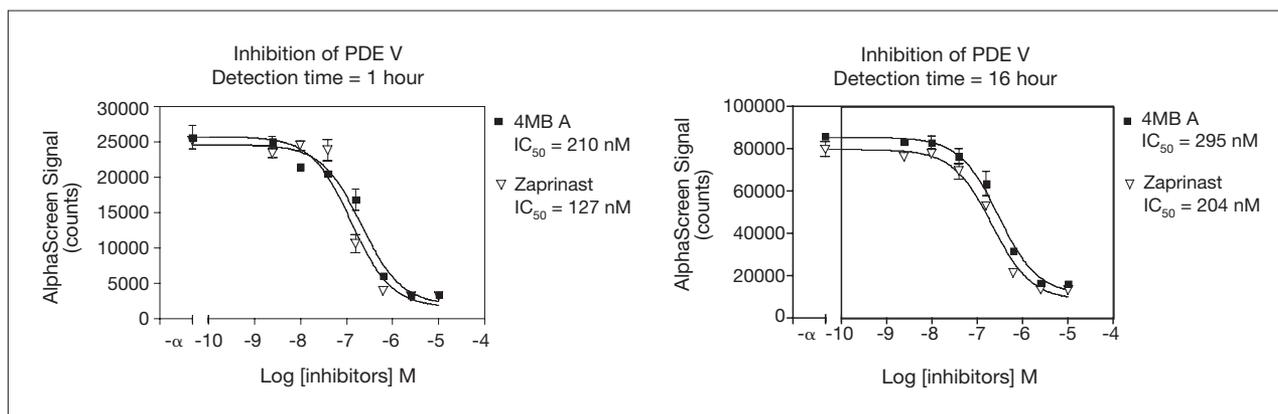


Figure 4. Inhibition Curves for PDE V

4) 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 a Z' value greater than 0.7 is considered excellent³. A preliminary assessment of the Z' value³ was conducted manually with two populations of 48 replicates to assess the robustness of the cGMP PDE assay in the 384 format. The two data sets were generated in the presence or absence of 10 μM Zaprinast inhibitor respectively. It is worth mentioning that Zaprinast was diluted in the PDE reaction buffer containing 5% DMSO (final concentration in 10 μL enzymatic reaction). Figure 5 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 obtained

suggesting that the assay is robust and suitable for compound screening and HTS campaigns.

Conclusion

The AlphaScreen cGMP detection assay which uses the cGMP probe and anti-cGMP antibody is very sensitive, allowing the detection of 10-20 fmoles of cGMP per well in 384-well microplates. 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 V. The optimized assay was shown to be robust and specific, combining a high level of sensitivity with cost-effectiveness properties.

References

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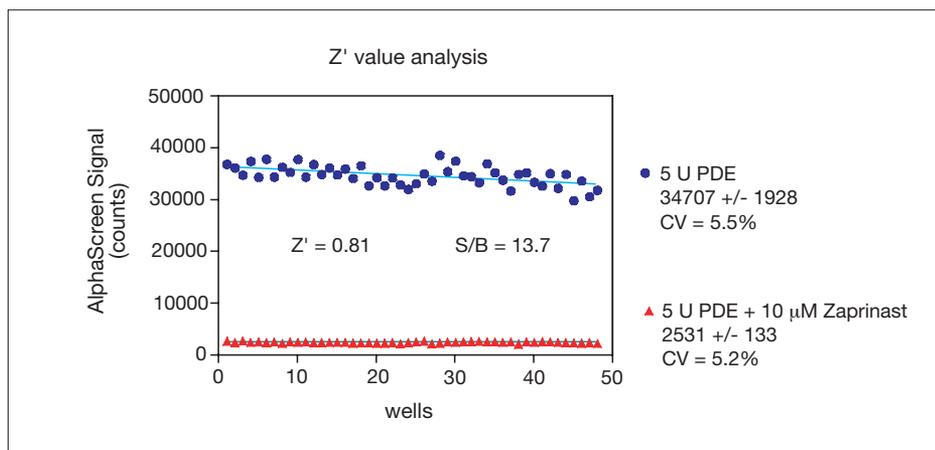


Figure 5. Screening validation of AlphaScreen cGMP PDE assay. Representative Z' graph for a 384 manual assay.