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Transcreener™ PDE Assay: Homogenous AMP and GMP Detection

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Introduction

The Transcreener™ HTS Assay Platform enables the screening of an entire group-transfer enzyme family using one set of detection reagents. This is accomplished by detection of the invariant product of these reactions (e.g.: ADP for Kinases and UDP for glycosyltransferases). The Transcreener™ PDE Assay is a universal, homogenous, competitive fluorescence polarization immunoassay that detects AMP and GMP produced by the cAMP and cGMP Phosphodiesterase (PDE) family using one reagent mix (Figure 1). This assay is sensitive and robust generating Z' > 0.5 at low substrate concentrations (nanomolar product). Additionally, a far-red tracer has been employed greatly reducing fluorescent and light scattering interference from test compounds and reagents. Here selective inhibition of PDE4A4, an established drug target for the treatment of inflammatory disease as well as depression, is demonstrated with the Transcreener™ PDE assay.

Figure 1. The Transcreener™ PDE Assay Measures AMP and GMP

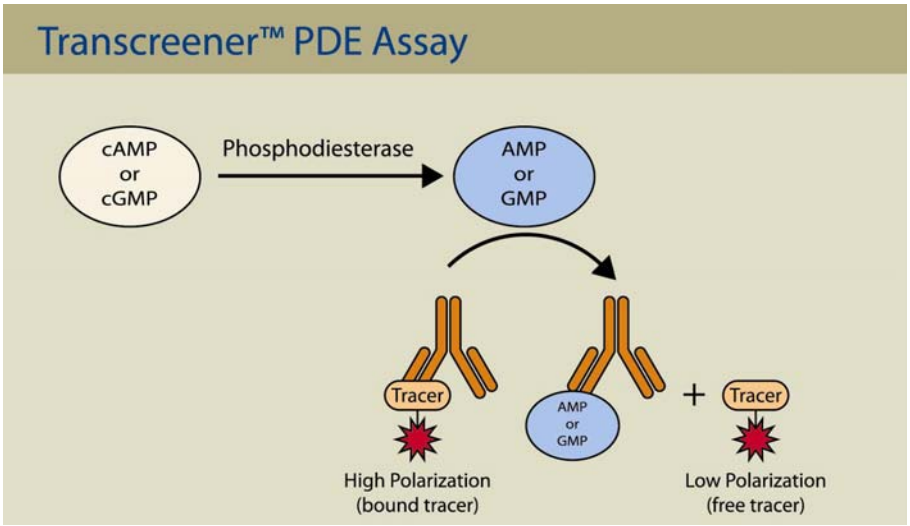
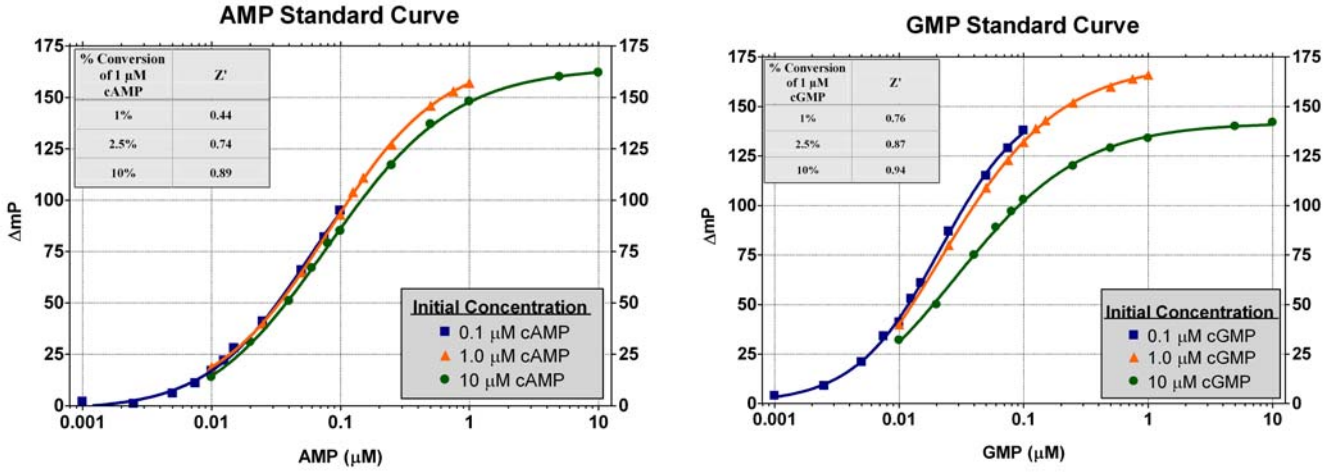
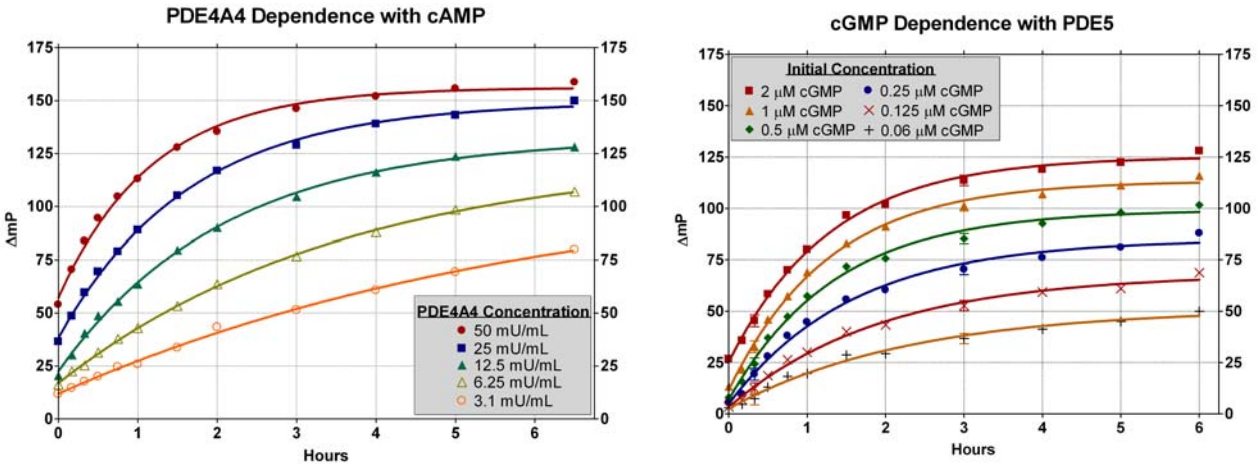


Figure 2. Robust, Sensitive AMP and GMP Detection in a Single Assay



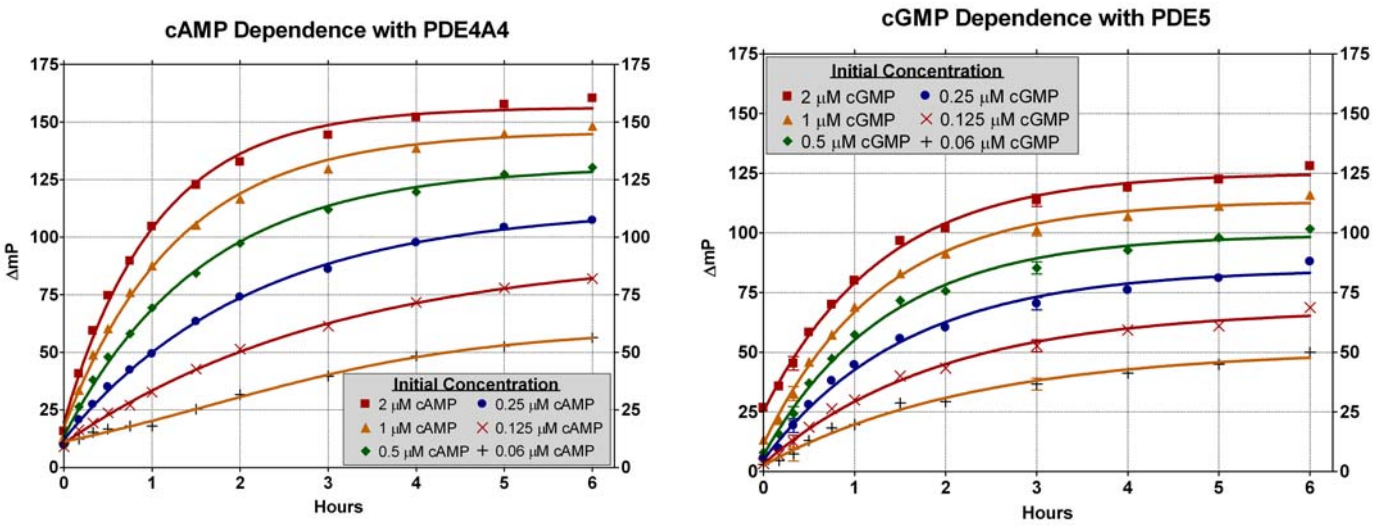
These standard curves mimic enzymatic catalysis of cAMP or cGMP to AMP or GMP. As cAMP or cGMP concentration decreases, AMP or GMP concentration increases concomitantly, maintaining a constant nucleotide concentration (e.g. 10% conversion of 10 µM cAMP equals 1 µM AMP and 9 µM cAMP). Each point on these standard curves was performed with 24 replicates in order to calculate Z' values. 30 µL reactions were performed in a standard PDE assay mix of 50 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 0.01% v/v Brij-35, 1% v/v DMSO, 20 mM EDTA, 4 nM PDE AlexaFluor® 633 tracer, and 180 µg/ml A(G)MP antibody. Polarization was measured with a Tecan Safire 2 (Ex_{635}/Em_{670}) after a 1 to 2 hour incubation at room temperature.

Figure 3. Product Formation is Enzyme Dependent for both PDE4A4 and PDE5



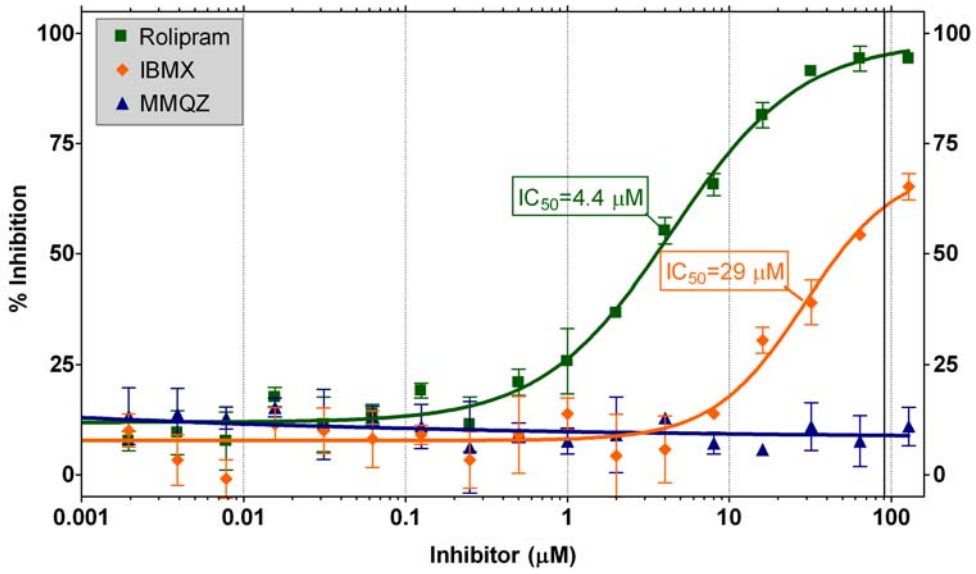
Enzyme dependent AMP and GMP production was demonstrated with PDE4A4 and PDE5, respectively, using one set of detection reagents. Assays were performed at 30°C in the standard PDE assay mix with the exclusion of EDTA (see figure 2). 1 µM substrate (cAMP for PDE4A4 and cGMP for PDE5) was used for the enzyme dependence experiments. PDE4A4 and PDE5 did not show significant activity with cGMP and cAMP respectively. (Data not shown)

Figure 4. Product Formation is Substrate Dependent for both cAMP and cGMP



Substrate dependent AMP and GMP production was demonstrated with 25 mU/mL of PDE4A4 and PDE5, respectively, using one set of detection reagents. Assays were performed at 30°C in the standard PDE assay mix (see figure 2) with the exclusion of EDTA. PDE4A4 and PDE5 did not show significant activity with cGMP or cAMP, respectively. (Data not shown)

Figure 5. Selective Inhibition of PDE4A4



Selective inhibition of PDE4A4 was demonstrated with the PDE4A4 specific inhibitor Rolipram and the broad PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX). Apparent IC_{50} values for Rolipram and IBMX were 4.4 µM and 29.1 µM respectively. No inhibition was observed with the PDE5-specific inhibitor 4-[{3', 4'-(Methylene-dioxy)benzyl]amino}-6-methoxyquinazoline (MMQZ). 1 µM cAMP and 25 mU/mL PDE4A4 were used. The PDE reaction was incubated at 30°C for 2 hours. 20 mM EDTA was added to stop the PDE reaction.

Conclusions:

1. Proof of concept has been established for a single, highly flexible and robust HTS assay for both cAMP and cGMP phosphodiesterases.

2. One reagent mix enables detection of nanomolar levels of AMP and GMP. Initial cAMP or cGMP concentrations from 0.1 to 10 µM may be used with one reaction mix. Slight formulation adjustment allows use of higher initial concentrations of cAMP or cGMP.

3. Z'-Factors of >0.5 are obtained with >7.5 nM GMP or 25 nM AMP, facilitating the study of most PDEs near their K_m under initial velocity conditions.

4. Enzyme, substrate, and time dependent AMP and GMP production was demonstrated with PDE4A4 and PDE5 respectively. PDE4A4 preferentially utilized cAMP and PDE5 preferentially utilized cGMP.

5. Dose-dependent inhibition of PDE4A4 was observed with Rolipram and 3-isobutyl-1-methylx anthine (IBMX) with respective IC_{50} values of 4.4 and 29.1 µM. The PDE5-specific inhibitor, MMQZ, did not inhibit PDE4A4.

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