

Homogenous Luminescent HTS-formatted Technologies for cAMP- and cGMP-Dependent Phosphodiesterases

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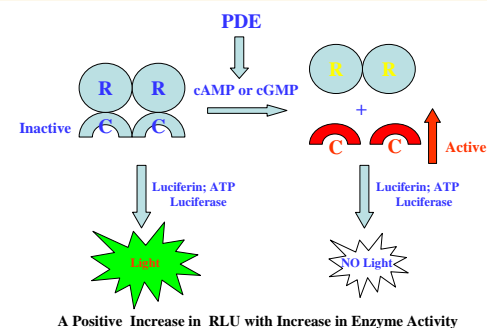
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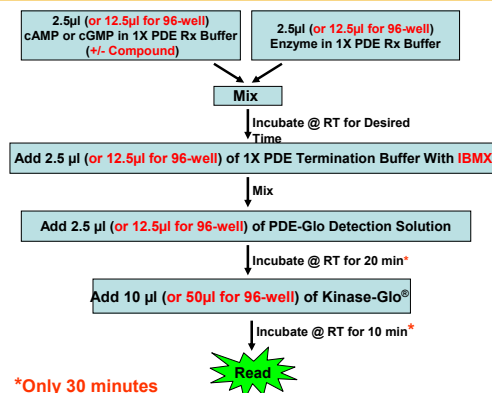
1. Abstract

Cyclic nucleotide phosphodiesterases (PDEs) comprise eleven different gene families that are encoded by at least 21 different genes and accounting for at least 55 isoforms. They have been implicated in controlling specific cellular functions including Cardiovascular, pulmonary, and inflammatory diseases and they play potential role as targets for treatment of cancer and some other diseases. Inhibition of PDEs was shown to induce chronic lymphocytic leukemia (CLL) apoptosis and more evidence support targeting PDEs for treatments of several types of cancer. Thus, the development of novel isoform-specific inhibitors may be useful for the development of therapeutic agents against cancer. We have developed a homogenous PDE-Glo™ Phosphodiesterase Assay for high-throughput screening (HTS) for novel inhibitors of PDE. The HTS formatted assays can be used to monitor the activity of cAMP-PDEs and cGMP-PDEs and can be carried out in two simple steps in 96-, 384-, or 1536-well formatted plates. The Assay is luminescent and thus it encounters minimal interference from fluorescent compounds and the robustness of the assays are indicated by their remarkably high Z' value (>0.8). In summary, the PDE-Glo luminescent assay can be used for screening libraries of compounds targeting in PDEs that might be developed into potential drugs for treatment of cancer and some other diseases.

2. Principle of PDE-Glo Phosphodiesterase Assay



3. Protocol for PDE-Glo Phosphodiesterase Assay (96- and LV 384-Well)



4. cNMP Titration Using PDE-Glo Assay

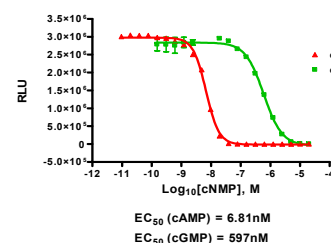


Figure 1. A PDE-Glo Assay performed at room temperature. The reaction was performed in a solid white 96 well plate using the indicated amount of cNMP. Luminescence was read on a Veritas (Turner Biosystems) luminescent plate reader (counts/sec). All data points are the average of two determinations and error bars are +/- standard deviation.

5. Bovine Brain PDE Titration Using cAMP and cGMP Substrates

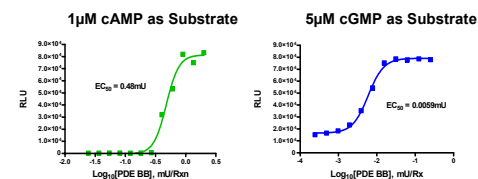


Figure 2. Titration of 3', 5'-cyclic-nucleotide-specific phosphodiesterase from bovine brain (Sigma-Aldrich, # P9529) using automation in PDE-Glo Assay. The PDE-Glo Assay was performed with the CyBio CyBio®-Well 384/1536 Pipetting System in a low-volume 384 plate using 1µM cAMP substrate (Left Panel) or 5µM cGMP, and the indicated amount of PDE bovine brain (Right Panel). Reactions were incubated for 5-minutes (cAMP) or 15 min (cGMP) at room temperature. Data analysis was performed with GraphPad Prism™ version 4.02. Each point represents an average of 4 replicates.

6. IBMX Titration with Bovine Brain PDE Using cAMP and cGMP Substrates

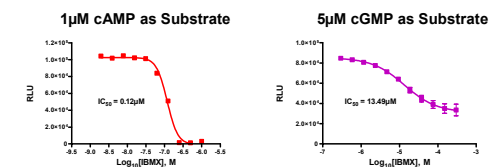


Figure 3. IC₅₀ for IBMX with PDE bovine brain using automation in PDE-Glo Phosphodiesterase Assay. The PDE-Glo Assay was performed with the CyBio CyBio®-Well 384/1536 Pipetting System in a low-volume 384 plate using 1µM of PDE bovine brain, 1µM cAMP substrate, and the indicated amount of IBMX (Left Panel) or 5µM cGMP substrate, and the indicated amount of IBMX (Right Panel). Substrate was added and the reactions were incubated for additional 5 minutes (cAMP) or 15-minutes (cGMP) at room temperature. Both data analysis were performed with GraphPad Prism™ version 4.02 for Windows using a sigmoidal dose-response (variable slope) equation.

7. PDE Type V: Enzyme and Inhibitor Titration

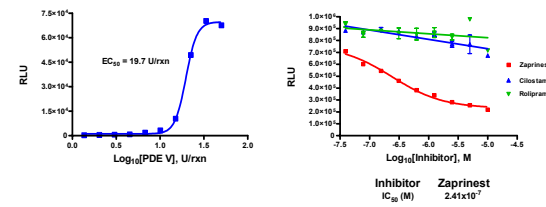


Figure 4. PDE Type V titration and PDE inhibitors titration using PDE-Glo Assay. Titration of PDE Type V was performed using the CyBio®-Well 384/1536 pipetting system (Cy Bio) in a standard white 384-well plate with 10µM cGMP as the substrate and the indicated amount of PDE V (Calbiochem, Cat.# 524715, Lot# D34976). Inhibitors titration was carried out using 15 U/well of PDE Type V, 5 µM cGMP and indicated concentrations of inhibitors. Each point represents an average of four replicates.

8. Other Examples of PDE (PDE 4B2 catalytic Domain & PDE 11A1) Using PDE Glo™ Luminescent Assay

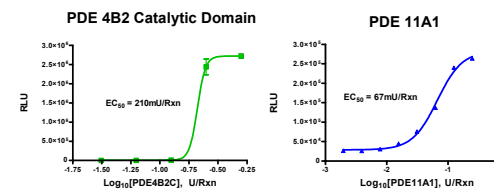


Figure 5. Titration of other PDEs using cAMP or cGMP as its substrate. Two different PDEs was performed using PDE-Glo Assay in solid white 96-well plate. PDE4B2 Catalytic Domain (Calbiochem, Cat.# 524732, Lot# B74999) using 1µM cAMP as substrate (Left Panel) and PDE11A1 (Calbiochem, Cat.# 524735, Lot# B72379) 10µM cGMP as substrate (Right Panel), and the indicated amount of PDEs were using in reactions. All data points were the average of two determinations and error bars are +/- standard deviation. Curve Fitting was performed using GraphPad Prism 4.02 Sigmoidal dose-response (variable slope) software.

9. Z' Factor Determination LV384 and 1536-well Formats

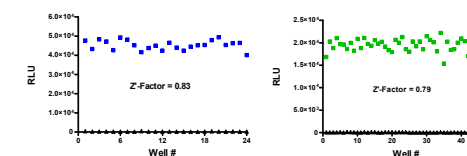


Figure 9. Z' factor determination using PDE bovine brain in low-volume 384 (Left Panel) and 1536-well formats (Right Panel) using automation in PDE-Glo Assay. The PDE-Glo Assay was performed with the CyBio CyBio®-Well 384/1536 Pipetting System in a low-volume 384 and a 1536-well plate using 1µM cAMP substrate per reaction, 1mU/rxn PDE bovine brain (LV384) or 0.4mU/rxn PDE bovine brain (1536). Reactions were incubated for 5-minutes at room temperature. 1µl each of PDE, substrate, Termination Buffer, and Glo Detection buffer were dispensed into the 1536-well plate, followed by the addition of 4µl of Kinase-Glo Reagent. Luminescence was measured using the BMG PHERAstar plate reader. Data shows results with PDE (higher RLU) versus no PDE (lower RLU).

10. LOPAC Screening with PDE-Glo Assay (with >20% Inhibition)

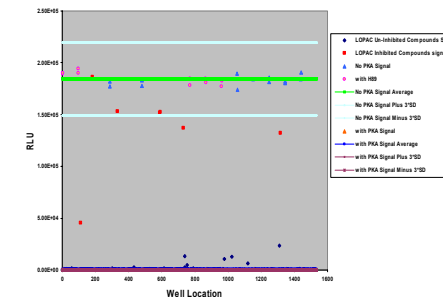


Figure 10. Automated HTS Using PDE-Glo Assay for LOPAC Library (Sigma): 384-Well white plate, 60µl total volume per well. Using PDE-Glo Assay (10µM of each compound). The Tecan Freedom Evo 200 with integrated TeMo was used. Luminescence was read on GENios Pro plate reader (0.5sec integration per well).

11. LOPAC Library Screen Results

Well Location	Compound	Usage	Inhibition
2 - F11	H89	PKA Inhibitor	100%
2 - H2	H9 Dihydrochloride	Protein Kinase Inhibitor most effective for PKA and PKG	25%
4 - D6	4-Chloro Mercuribenzoic Acid	Carboxy- & Aminopeptidase inhibitor	83%
7 - C2	(-) - Ephedrine Hemisulfate	Very weak β2-adrenergic agonist	82%
7 - G2	Ebselen	Potent Antioxidant, inhibits lipoxygenase and cyclooxygenase	83%
8 - B8	Hydroquinone	Arachidonate 12-Lipoxygenase inhibitor	74%
14 - D9	SCH-202676 Hydrobromide	Allosteric inhibitor of agonist and antagonist binding to GPCR. Inhibits a variety of GPCRs (opioid, muscarinic, adrenergic, dopaminergic receptors)	72%

12. Features of the PDE-Glo Phosphodiesterase Assay

- Universal: Both cAMP and cGMP Phosphodiesterases
- Homogenous, Nonradioactive and Antibody free
- Luminescent, Free of fluorescence interference
- HTS Formatted: (96-, 384-, and 1536-well Plates)
- Robust (Z'>0.8) and very sensitive to low cNMP Concentration
- Stable Reagents and Detection Signal

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