A Phosphodiesterase Assay using LANCE Technology: Simple, Flexible and Sensitive

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1 Abstract

Cyclic nucleotide phosphodiesterases (PDE) catalyze the degradation of cellular cAMP or cGMP. They regulate the localization, duration, and amplitude of cyclic nucleotide signaling within sub-cellular domains. PDEs are therefore important regulators of signal transduction mediated by these second messenger molecules which have become important drug targets for the treatment of several diseases, such as asthma, chronic obstructive pulmonary disease, and neurodegenerative diseases to name a few. PerkinElmer has a homogenous time-resolved fluorescence resonance energy transfer (TR-FRET) technology termed LANCE that can be used to monitor the activity or inhibition of PDEs. The assay can be easily setup using a cAMP specific antibody labeled with the dye, Alexa Fluor® 647, biotin-cAMP and streptavidin labeled with Europium (Eu-SA). As the complex of Eu-SA / biotin-cAMP / Alexa Fluor 647 labeled antibody is formed, an increase in signal is generated. When there is PDE activity, resulting in the degradation of the cyclic nucleotide, the complex is not formed and a decrease in signal is observed. Proof of concept data for several PDE's will be presented. The assay has been optimized for 384-well microplates, but would be amenable to 96- or 1536-well plates as well.

2 Introduction

Using the LANCE cAMP kit, a phosphodiesterase assay was developed. This competition based assay results in a positive signal being generated in the presence of increased PDE inhibition. The assay was formatted using the biotinylated cAMP as the substrate for the PDE. As the PDE activity was increased, the complex of Eu-SA / biotin-cAMP / Alexa Fluor 647 labeled antibody was disrupted and the signal was decreased. Assay performance was evaluted by analyzing several different PDE's, specific inhibitors to PDE4A1A, and determining optimal PDE incubation time and precision.

3 Materials

LANCE cAMP Kit – PerkinElmer, Inc. Product # AD0262
Enzyme Assay Buffer Components:
HBSS –Invitrogen Corp. Product # 14025-092 (1X solution)
HEPES – Invitrogen Corp. Product # 15630-080 (1 mol/L solution)
BSA – 7.5% Stabilizer PerkinElmer, Inc. Product # CR84-100
$MgCl_2$ – Fluka Product # 63020 (1 mol/L solution)
Enzymes:
- PDE – BPS Bioscience
- PDE2A - Product # 60020
- PDE3B - Product # 60031
- PDE4D2 - Product # 60043
- PDE4A1A - Product # 60040
- PDE7B - Product # 60071
- PDE8A1 - Product # 60080

Inhibitors:
- IBMX – Sigma-Aldrich, Inc. Product # 15879
- Rolipram - Sigma-Aldrich, Inc. Product # R6520
- RO-20-1724 – Biomol Product # EI-117

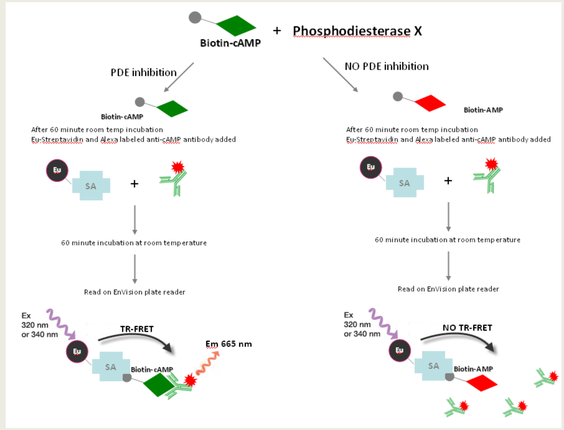
4 Reagent Preparation

Enzyme Buffer – 1X HBSS containing 5 mM HEPES, 0.1 % BSA, and 1.5 mM $MgCl_2$, pH 7.4
Enzyme – Example: PDE41A (BPS Bioscience Cat# 60040 0.2 mg/mL) –
Prepare serial dilutions in Enzyme Buffer
Biotinylated cAMP (LANCE cAMP kit) –
Prepare Intermediate Stock – 10 µL Stock + 20 µL Enzyme Buffer
Prepare Working Solution – 5 µL Intermediate Stock + 620 µL Enzyme Buffer
Detection Mix (LANCE cAMP kit) –
Prepare Intermediate Stock of Streptavidin Europium – 5 µL Stock + 85 µL Detection Buffer
Working Solution – 5 µL SA-Eu Intermediate Stock + 3 µL Alexa Fluor® 647 labeled Antibody + 615 µL Detection Buffer

5 Method

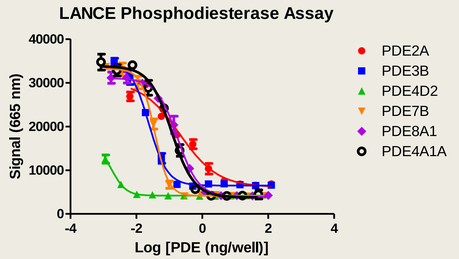
5 µL Biotinylated cAMP
5 µL PDE or 2.5 µL PDE and 2.5 µL Inhibitor
Incubate 1 hour at room temperature
10 µL Detection Mix
Incubate 1 hour at room temperature

6 Schematic



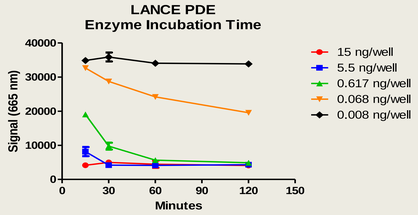
The schematic depicts the action of the PDE enzyme utilizing the LANCE technology. When there is Inhibition of the PDE, the antibody binds to the b-cAMP and the complex is formed allowing TR-FRET to occur. Alternately, when there is no PDE inhibition, the PDE degrades the b-cAMP to b-**AMP**. The antibody does not recognize the AMP and therefore, the complex is not formed and TR-FRET does not occur.

7 Results

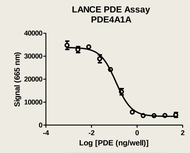


	PDE2A	PDE3B	PDE4D2	PDE7B	PDE8A1	PDE4A1A
Best-fit values						
Bottom	6116	6481	4181	4128	3892	3782
Top	30397	34958	15591	33190	31041	33781
LogEC50	-0.6830	-1.624	-2.730	-1.443	-0.7532	-0.9276
HillSlope	-0.7507	-1.547	-2.042	-2.096	-1.356	-1.277
EC50	0.2075	0.02375	0.001861	0.03608	0.1765	0.1181
Span	24281	28477	11411	29062	27150	30000

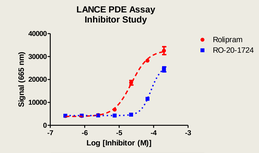
Several Phosphodiesterase enzymes were evaluated. Data suggests that each PDE enzyme converted cAMP to AMP resulting in a decrease in signal.



Varying levels of PDE4A1A enzyme were evaluated for optimal reaction time. The optimal incubation time depended on the enzyme concentration selected.

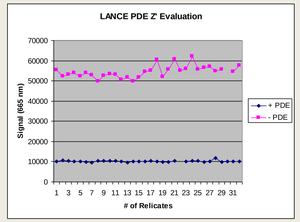


	PDE4A1A
Best-fit values	
Bottom	3782
Top	33781
LogEC50	-0.9276
HillSlope	-1.277
EC50	0.1181
Span	30000



	Rolipram	RO-20-1724
Best-fit values		
Bottom	3856	4242
Top	32741	25854
LogEC50	-4.644	-4.079
HillSlope	1.739	2.995
EC50	2.272e-005	8.344e-005
Span	28885	21612

PDE4A1A was chosen to evaluate selective inhibitors. Two specific PDE inhibitors were evaluated, Rolipram and RO-20-1724. Results suggest that both inhibitors performed as expected.



A precision study was performed to determine assay performance. A Z' of 0.78 was obtained when measuring with and without PDE4A1A in the well.

8 Summary

Using the LANCE cAMP kit (PerkinElmer, Inc. Product #AD0262), a phosphodiesterase assay was developed. The assay was formatted using the biotinylated cAMP as the substrate for the PDE; however, it is plausible that biotinylated cGMP could also be a viable substrate. This data suggests that phosphodiesterase assays can be successfully performed with ease and optimal performance.

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