

Phosphodiesterase Assays for High Throughput Screening

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Introduction

cAMP is an important second messenger regulating diverse function such as cell shape, protein phosphorylation, gene transcription, etc. As much as 10 subtypes of adenylyl cyclase are capable of converting ATP to cAMP. Breakdown of cAMP is mediated through another class of enzymes known as phosphodiesterases (PDEs). These enzymes can be classified according to their specificity for cAMP and cGMP. There exist PDEs that only hydrolyze cAMP or cGMP, as well as other subtypes that hydrolyze both cyclic nucleotides.

Detection of the activity of cAMP converting PDEs has classically been performed using radioactive methods. In this study, we have compared the use of a non-radioactive method based on fluorescence polarization to the classically used radioactive FlashPlate® method.

Both methods rely on an antibody recognizing labeled cAMP (tracer). In the FlashPlate method, [¹²⁵I]cAMP is used, while fluorescein-labeled cAMP is used in the fluorescence polarization method. When the tracer is cleaved by PDE, the antibodies no longer recognize the AMP generated from this reaction leading to a decrease in signal. For an overview of the principle, see Figure 1A and 1B.

Our study indicates that both methods can be set up as homogenous assays, which can be easily miniaturized. Hence, both techniques are ideal for use in high throughput screening.

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Basic Principle Fluorescence Polarization

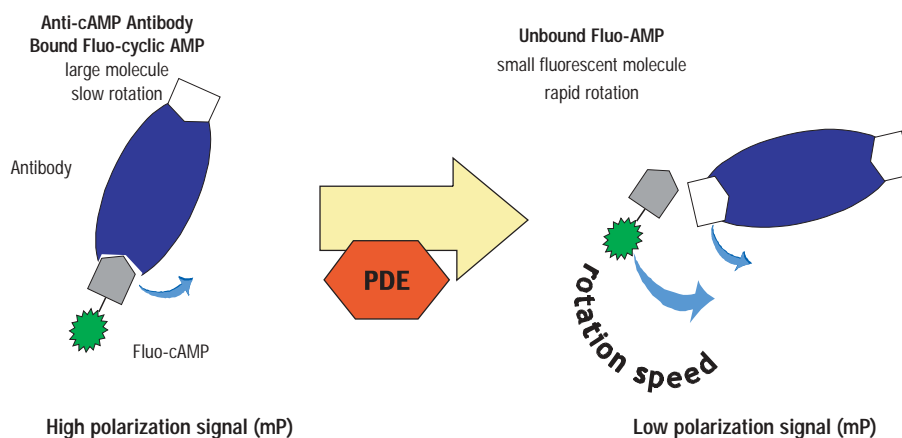


Figure 1A.

- Cleavage of Fluo-cyclic AMP by PDE leads to Fluo-AMP which cannot be recognized by the antibody
- PDE activity produces lower polarization signal

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Basic Principle FlashPlate Assay

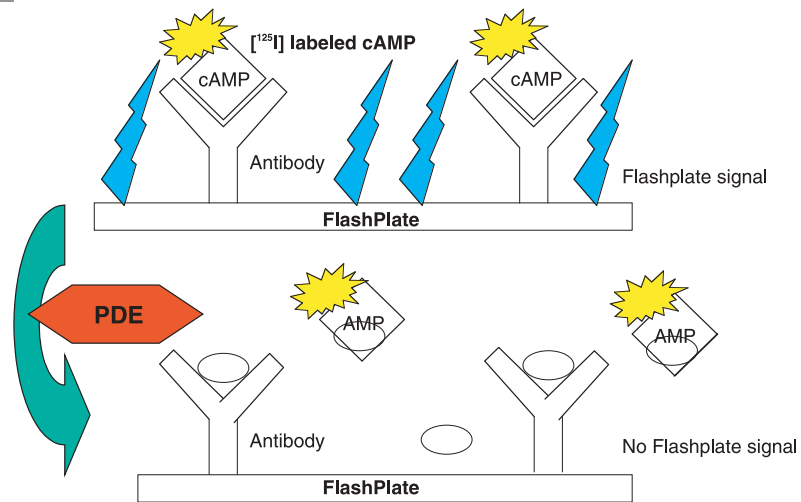


Figure 1B.

- PDE activity hydrolyzes [125I] labeled cyclic AMP to [125I] labeled AMP, which is no longer recognized by antibody => no scintillation proximity signal

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Critical Materials

- [FP]^{2™} Fluorescence Polarization cAMP Assay (PerkinElmer Catalog # FPA202) (used for PDE assays)
 - cAMP Standard
 - cAMP Antibody
 - Fluo-cAMP Tracer
 - Detection Buffer
- Microtiter plates (black, Corning Costar® 384-well microplates #3710)
- Adenylyl Cyclase Activation FlashPlate Assay (PerkinElmer Catalog # SMP701) (used for PDE assays)
 - cAMP Standard
 - [125I]cAMP Tracer
 - Detection Buffer
 - Adenylyl Cyclase Activation FlashPlate
- Phosphodiesterase I (Sigma, Cat# P6903)

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Methods

[FP]² Fluorescence Polarization Phosphodiesterase Assay

1. *Standard Curve*

- 10 µL of Stimulation Mix (contains antibody at 1:10, prepared as described in manual)
- 10 µL of cAMP diluted in Detection Buffer (a series of dilutions)
- 20 µL of Detection Mix (containing Fluo-cAMP at 5 nM final)
- Incubate for 60 minutes at room temperature
- Read on a Fluorescence Polarization Reader

2. *Phosphodiesterase Activity (PDE)*

- 10 µL of Stimulation Mix (containing antibody)
- 10 µL of PDE diluted in Detection Buffer (a series of dilutions)
- 20 µL of Detection Mix (containing four concentrations of Fluo-cAMP)
- Incubate for 60 minutes at room temperature
- Read on a Fluorescence Polarization Reader

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Methods (cont.)

Phosphodiesterase FlashPlate Assay

1. *Standard Curve*

- 25 µL of cAMP diluted in Stimulation Buffer (a series of dilutions)
- 25 µL of Detection Mix (containing [¹²⁵I]cAMP)
- Incubate for 2 hours at room temperature
- Read on a microplate scintillation counter

2. *Phosphodiesterase Activity (PDE)*

- 25 µL of PDE diluted in Stimulation Buffer (a series of dilutions)
- 25 µL of Detection Mix (containing [¹²⁵I]cAMP)
- Incubate for 2 hours at room temperature
- Read on a microplate scintillation counter

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Results

[FP]² Fluorescence Polarization PDE Assay

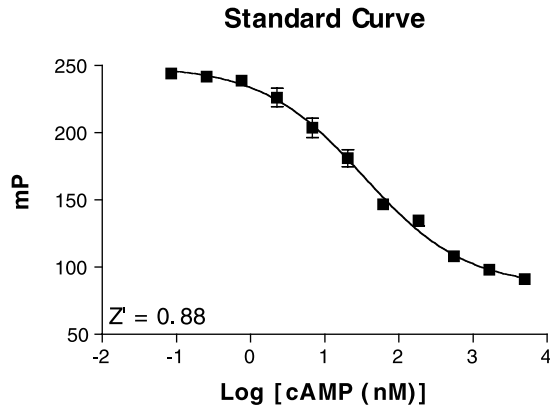
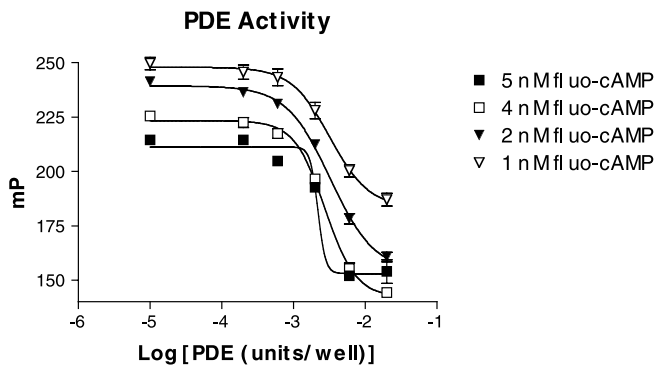


Figure 2. cAMP Competition Curve

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Results (cont.)

[FP]² Fluorescence Polarization PDE Assay



	5 nM fluo-cAMP	4 nM fluo-cAMP	2 nM fluo-cAMP	1 nM fluo-cAMP
EC ₅₀	0.002195	0.002756	0.003255	0.003207

Z' = 0.73 Z' = 0.78 Z' = 0.80 Z' = 0.53

Figure 3. PDE Activity

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Results Phosphodiesterase FlashPlate Assay

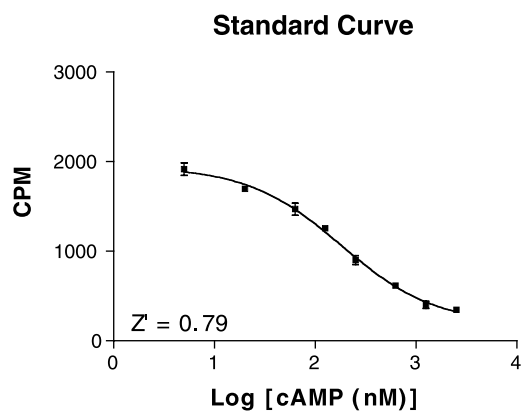
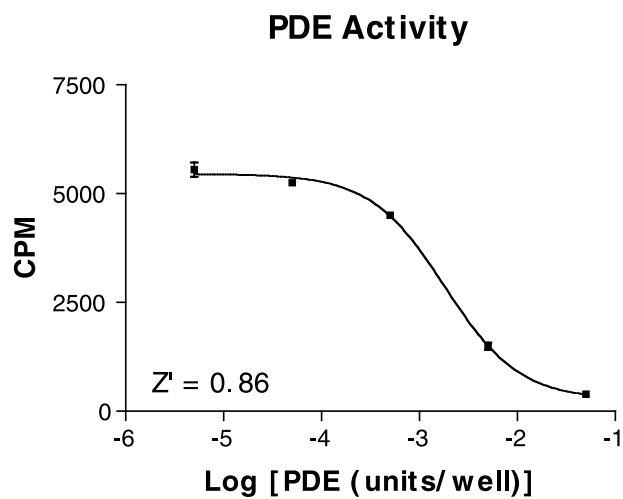


Figure 4. cAMP Competition Curve

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Results (Cont.) Phosphodiesterase FlashPlate Assay



	[¹²⁵ I]cAMP
EC ₅₀	0.001811

Figure 5. PDE Activity

Conclusion

[FP]² Fluorescence Polarization Phosphodiesterase Assay and the Phosphodiesterase FlashPlate Assay can be used with purified PDE with excellent performance.

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