APPLICATION NOTE

IMAP phosphodiesterase assays on SpectraMax Multi-Mode Microplate Readers

Introduction

IMAP® Technology from Molecular Devices enables rapid, homogeneous, and non-radioactive assay of kinases, phosphatases, and phosphodiesterases and is suited for both assay development and high-throughput screening. IMAP assays are based on binding of phosphate to immobilized metal coordination complexes on nanoparticles. When IMAP binding entities bind to phosphorylated substrate, molecular motion of the peptide is altered, and fluorescence polarization (FP) for the fluorescent label attached to the peptide increases (Figure 1, left). In a TR-FRET version of the assay, the inclusion of a Terbium (Tb) donor enables a fluorescent energy transfer to occur when phosphorylated substrate is present (Figure 1, right). This assay is detected in a time-resolved mode, which virtually eliminates fluorescence interference from assay components or compounds in a screen. TR-FRET also offers flexibility in substrate size and concentration.

Cyclic nucleotide phosphodiesterases (PDEs) are a group of enzymes that degrade the phosphodiester bond of cAMP and cGMP, second messengers that are involved in a variety of biological processes. They have emerged as a key class of druggable targets due to their clinical significance in areas including heart disease, dementia, depression, and others. In this application we demonstrate how PDE enzyme dilution and inhibition curves are performed with IMAP Technology using the SpectraMax® i3, SpectraMax® Paradigm®, SpectraMax® M5, and FlexStation® 3 Multi-Mode Microplate Readers with SoftMax® Pro Software.

Benefits

- **Non-antibody-based platform**
- **Sensitive and stable**
- **Complete, homogeneous assay system**
- **FP and TR-FRET options**

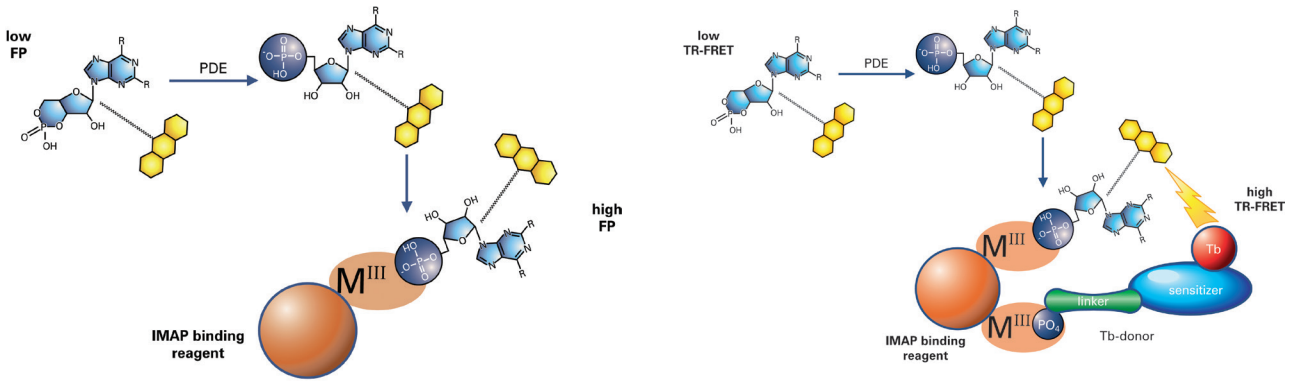


Figure 1. IMAP FP and TR-FRET phosphodiesterase assay principle. A phosphodiesterase reaction is performed using a fluorescent-labeled substrate. Binding Solution containing large M(III)-based nanoparticles is then added. In the FP readout **(left)**, the small phosphorylated fluorescent substrate binds to the large nanoparticles, which reduces the rotational speed of the substrate and thus increases its fluorescence polarization. In the TR-FRET readout **(right)**, phosphorylated substrate and Tb donor both bind to the nanoparticle, bringing the Tb donor in close proximity to the fluorescein acceptor on the substrate and enabling FRET.

Materials

- IMAP FP PDE Evaluation Kit (Molecular Devices P/N R8175)
- IMAP TR-FRET PDE Evaluation Kit (Molecular Devices P/N R8176)
  - IMAP Progressive Binding Reagent
  - IMAP Progressive Binding Buffer A (5X)
  - IMAP Progressive Binding Buffer B (5X)
  - TR-FRET Tb Donor
  - IMAP Reaction Buffer with 0.01% Tween-20 (5X)
  - Fluorescein-labeled cGMP Substrate (Molecular Devices P/N R7507)
- Phosphodiesterase 1, 3', 5'-cyclic-nucleotide-specific from bovine brain (Sigma P/N P9529)
- Calmodulin (Sigma P/N P1431)
- Calcium chloride (major laboratory suppliers)
- DL-Dithiothreitol (DTT) (major laboratory suppliers)
- 384-well polystyrene black (for FP) and white (for TR-FRET) microplates (e.g., Corning P/N 3573 and 3572)

Methods

Enzyme dilution series

Complete reaction buffer (CRB) was prepared with 1 mM DTT, 2500 U/mL Calmodulin, and 2.5 mM CaCl2. Fluorescein-labeled cGMP substrate was diluted in CRB to make a 200 nM (2X) working concentration. Optimal substrate concentration and other assay parameters were determined previously (IMAP Assay Archive). A 1:3 dilution series of PDE1 enzyme, starting at 0.002 U/mL, was prepared as 2X working dilutions in CRB. Enzyme reactions were assembled in quadruplicate at a volume of 20 µL per well in a black (FP) or white (TR-FRET) 384-well plate with the following additions:

- 10 µL of 2X enzyme dilution or no-enzyme (CRB)
- 10 µL of 2X substrate solution

For buffer-only controls, 20 µL CRB was added to wells. Assays were incubated at room temperature for one hour.

Parameter	Fluorescence polarization	TR-FRET
Read type	Endpoint	Endpoint
Read mode	FP	TR-FRET
Detection cartridge	FP-FLUO Ex 485 nm/Em 535 nm	TR-FRET B/G (custom) Wavelength 1: Ex 340 nm/Em 490 nm Wavelength 2: Ex 340 nm/Em 520 nm
Plate type	384 Well Costar black	384 Well Costar black*
Read area	[User-defined]	[User-defined]
PMT and Optics	Integration time: 100 ms Read from Top Read height 4.27 mm	Integration time: 1 ms Excitation time: 0.05 ms Number of pulses: 100 Meas. Delay: 0.2 ms Read from Top Read height 6.02 mm

*This plate definition in SoftMax Pro Software is the same for black and white plates.

Table 1. SpectraMax i3 reader settings for IMAP PDE assays.

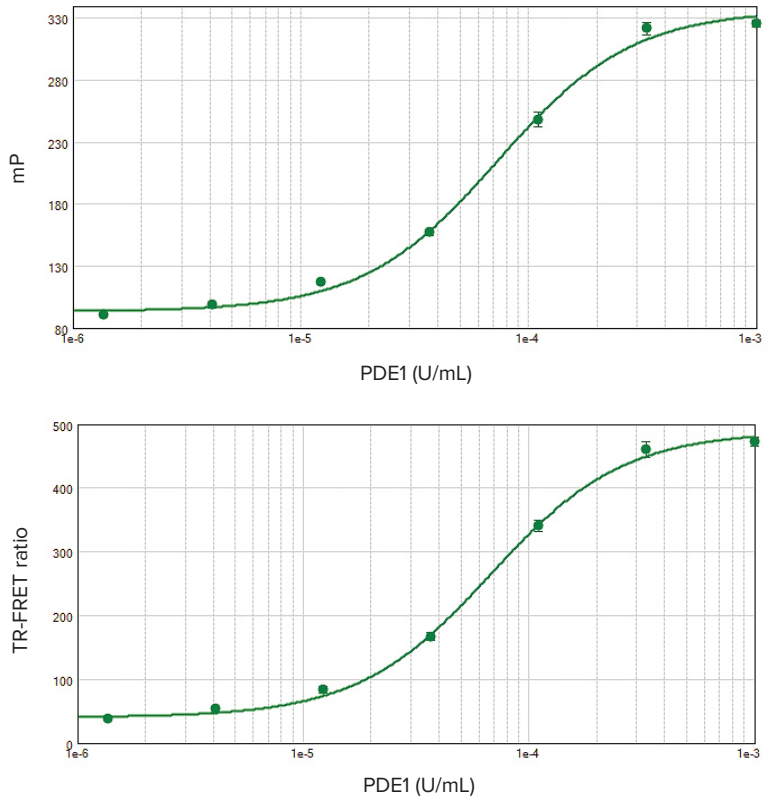


Figure 2. EC_{50} curves for IMAP FP and TR-FRET readouts. Top: FP; EC_{50} value was 7.3 x 10^{-5} units/mL with a Z' factor of 0.95. Bottom: TR-FRET; EC_{50} value was 6.8 x 10^{-5} units/mL with a Z' factor of 0.92.

Inhibitor assays

The PDE1 inhibitors 8-MM-IBMX and zaprinast were serially diluted in CRB at 4X working concentrations. Both series started at 200 µM (final in assay) and were diluted 1:3. PDE1 enzyme with EC_{80} concentration of 1.3 x 10^{-4} U/mL was prepared as a 4X working solution. Reactions were assembled as follows:

- 5 µL of 4X inhibitor or CRB for controls
- 5 µL of 4X enzyme
- 10 µL of 2X substrate solution

Assays were incubated at room temperature for one hour.

Binding reaction

IMAP Binding Solution specific to each assay detection mode was prepared. For FP, Binding Solution contained 75% Buffer A, 25% Buffer B, and 1:600 Binding Reagent. For TR-FRET, it contained 60% Buffer A, 40% Buffer B, 1:800 Binding Reagent, and 1:400 Tb Donor. Buffer-only controls received Binding Solution minus Tb Donor. 60 µL of the appropriate Binding Solution was added to each assay well, and assays were incubated for one hour (FP) or overnight (TR-FRET).

Detection

At the completion of the binding step, assay plates were read on the SpectraMax i3 and SpectraMax Paradigm readers using the settings in Table 1. All data were acquired and analyzed using SoftMax Pro Software. Preconfigured software protocols containing optimized settings and analysis parameters for each assay were used to simplify calculation of results. The Read Height Adjustment procedure was run prior to reading the plate to ensure optimal read height for each assay.

Results

Performing an enzyme dilution series verifies the enzyme and assay performance and allows calculation of the EC_{80} concentration of enzyme that will be used for inhibitor screening assays. PDE1 enzyme dilution series were performed using IMAP Technology in both FP and TR-FRET assay modes. EC_{50} values agreed very closely for both assay types (Figure 2) and were similar to previously published values. The Z' factor calculated from positive and negative controls was greater than 0.9 for each assay, indicating robust performance suitable for high-throughput screening. Inhibitor assays yielded similar IC_{50} values for both FP and TR-FRET assay readouts (Figure 3).

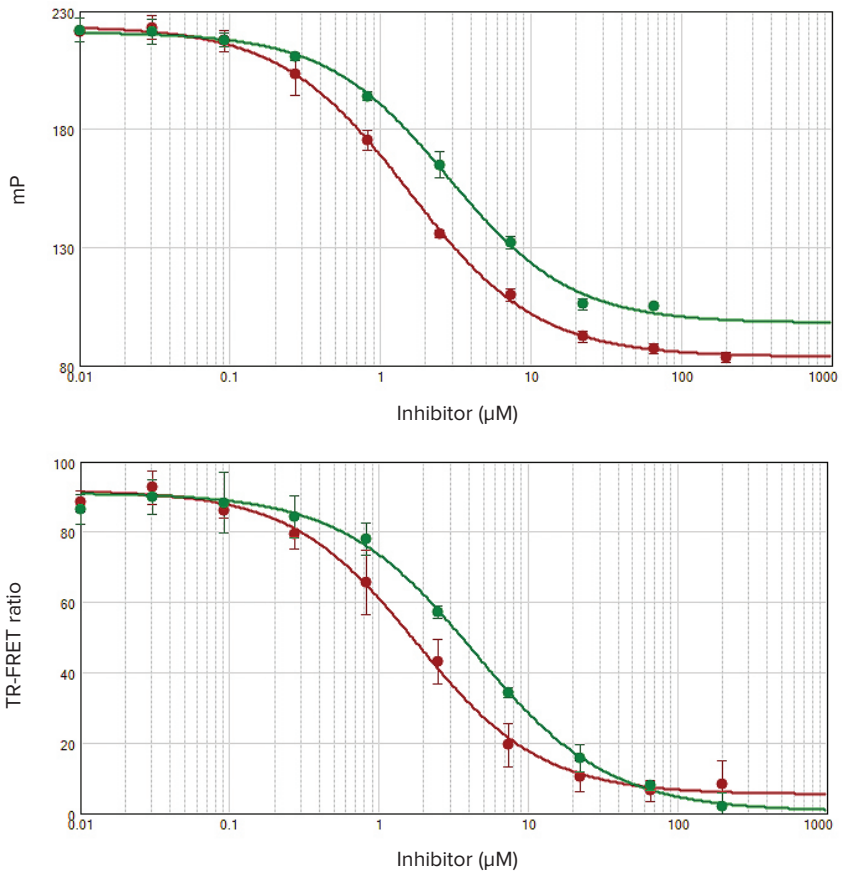


Figure 3. IC_{50} curves for IMAP FP and TR-FRET readouts. Top: FP. **Bottom:** TR-FRET. On each plot, IC_{50} curves for IBMX are in red and zaprinast are in green. For 8-MM-IBMX, IC_{50} values of 1.55 µM (FP) and 1.77 µM (TR-FRET) were obtained. For zaprinast, IC_{50} values were 2.83 µM (FP) and 4.35 µM (TR-FRET). These IC_{50} values are consistent with previously published results[1].

Conclusion

IMAP Technology offers a homogeneous, non-antibody-based platform for screening kinases, phosphatases, and phosphodiesterases. Stable and robust, it is amenable to miniaturization and yields high-quality data with excellent Z' factor values, making it suitable for inhibitor screening. Unlike some other methods, IMAP measures direct binding for more relevant results. IMAP TR-FRET assays also enable direct Km determination.

IMAP assays can be performed on systems shown on the next page. Data presented here were generated with the SpectraMax i3 reader. Similar results were obtained with the SpectraMax Paradigm reader, which offers the added advantage of fast, dual-PMT detection. SoftMax Pro Software with preconfigured assay protocols simplifies the workflow, from data acquisition to analysis. Molecular Devices offers a complete IMAP screening solution, including an IMAP Assay Archive with reference data on over 120 different enzymes, validated protocols, kits, and substrates[2].

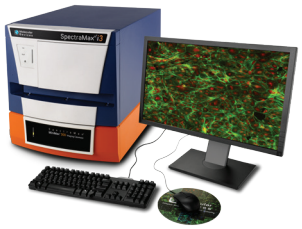
References

1. Ahn, HS, Bercovici, A, Boykow, G, et al. (1997). Potent tetracyclic guanine inhibitors of PDE1 and PDE5 cyclic guanosine monophosphate phosphodiesterases with oral antihypertensive activity. *J. Med Chem.* 40, 2196-2210.
2. IMAP Assay Archive, http://support.moleculardevices.com/assayarchive/index.html.

Ordering information		
Reagent	Amount	Part number
IMAP FP PDE Evaluation Kit	800 dp*	R8175
IMAP TR-FRET PDE Evaluation Kit	800 dp	R8176
IMAP FP Screening Express w/Progressive Binding System	8000 dp	R8127
IMAP TR-FRET Screening Express w/Progressive Binding System	8000 dp	R8160
cAMP PDE Substrate	20 nmol 120 nmol	R7505 R7506
cGMP PDE Substrate	20 nmol 120 nmol	R7507 R7508

* Data points

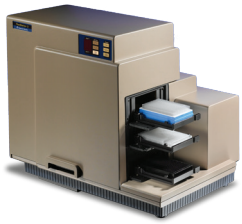
Compatible with these Molecular Devices systems



SpectraMax i3 Multi-Mode Microplate Reader

SpectraMax Paradigm Multi-Mode Microplate Reader

SpectraMax M5 Multi-Mode Microplate Reader



FlexStation 3 Multi-Mode Microplate Reader

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