# IMAP TR-FRET Phosphodiesterase Evaluation Assay Kit

## With Progressive Binding System

The IMAP® TR-FRET Phosphodiesterase Assay Kit is designed to evaluate activity of phosphodiesterases (PDEs) that use cyclic-AMP (cAMP) or cyclic-GMP (cGMP) as substrate. This kit uses IMAP technology for non-antibody based TR-FRET detection of enzyme activity and provides FAM-labeled derivatives of cAMP and cGMP as substrates. This assay is a simple, homogeneous mix and read procedure that allows accurate determination of PDE activity. Molecular Devices offers an IMAP Fluorescence Polarization (FP) Assay Kit for PDE's.

**Available Kit**

| | Data Points | Kit |
|---|---|---|
| IMAP TR-FRET Phosphodiesterase Evaluation Assay Kit | 800 (20 µL reactions) | R8176 |

### Contents




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Patents: http://www.moleculardevices.com/patents

Product manufactured by Molecular Devices, LLC.
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Molecular Devices, LLC is ISO 9001 registered.

# Chapter 1: About the Progressive Binding System

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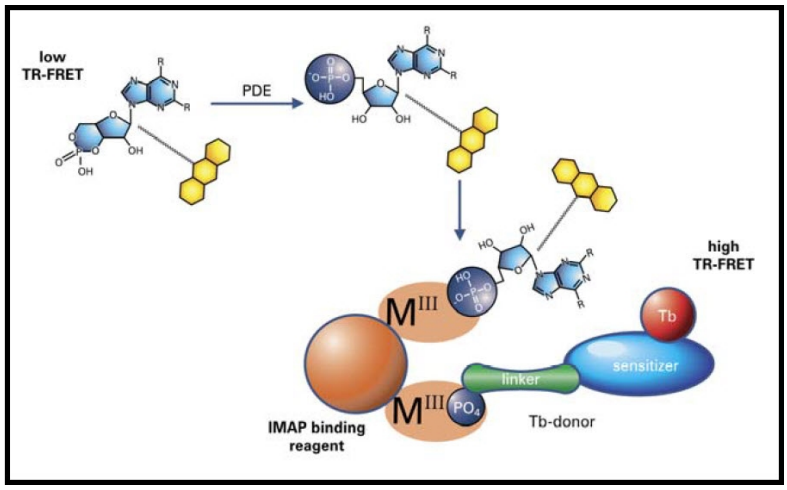
Use the IMAP Buffer Kits that are based on IMAP's antibody-free detection of phosphorylation in conjunction with fluorescent labeled substrates and enzymes to evaluate enzyme activity of kinases, phosphatases, and phosphodiesterases. The assay is a simple, homogeneous mix and read procedure that allows accurate determination of enzyme activity. The Progressive Binding System is adjustable to allow you to determine maximum performance for each application. The IMAP Progressive Binding System kits described in this document are designed for detection in TR-FRET Mode.

## Assay Principle

The IMAP technology is based on the high affinity binding of phosphate by immobilized metal ($M^{III}$) coordination complexes on nanoparticles. This IMAP Binding Reagent complexes with phosphate groups on phosphopeptides generated in a kinase reaction or on a nucleotide monophosphate generated from cyclic nucleotides (cAMP/cGMP) through phosphodiesterases. Such binding causes the fluorophore on the product to come in close proximity to the Tb-Donor also binding to the binding entities and fluorescence resonance energy transfer (FRET) is generated upon excitation. Due to the long lifetime of the Tb-Donor this can be measured in a time-resolved mode, which further reduces the background of the assay.




**Principle of the IMAP TR FRET PDE Assay System**

## Applications

The IMAP PDE Evaluation kit is designed for use in biochemical in-vitro assays of phosphodiesterase activity. This kit provides both FAM-labeled cAMP and cGMP as possible substrates to evaluate the optimal configuration for the assay. This technology is especially suited for high throughput screening applications, and you should use the IMAP Binding System Component (8000 data points Screening Express Buffer System: part no R8160, FAM-cAMP: part no R7505, FAM-cGMP: part no R7507). For larger screening amounts inquire about the Molecular Devices volume discount program.

# Chapter 2: Materials and Equipment

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## Kit Components

**Components of the IMAP TR-FRET Progressive Binding System**

| Reagent | Description |
|---|---|
| IMAP Progressive Binding Buffer A | Baseline binding buffer |
| IMAP Progressive Binding Buffer B | Affects background by reducing, or blocking, the non-phosphate-based binding of the fluorescent substrate to the Binding Reagent. |
| IMAP Progressive Binding Reagent | Introduces the phosphate binding entities. This Binding Reagent specifically binds to phosphate residues via a coordinate covalent complex bond. |
| IMAP TR-FRET Donor (=Tb based Phospho conjugate) | Enables the TR-FRET read-out based on the close proximity of Tb-Donor and fluorophore on phosphorylated substrate by binding both, even under very stringent Binding Buffer conditions, to the Binding entities. |

**Components of the IMAP Phosphodiesterase Evaluation Kit**

| Reagent | Quantity | Description |
|---|---|---|
| IMAP Progressive Binding Reagent | 150 µL | One vial, store 4°C<br>**Do not freeze the Binding Reagent** |
| IMAP Progressive Binding Buffer A (5x) | 12 mL | One bottle, store 4°C |
| IMAP Progressive Binding Buffer B (5x) | 12 mL | One bottle, store 4°C |
| TR-FRET Tb Donor | lyophilized | One vial, store 4°C |
| IMAP Reaction Buffer with BSA (5x)* | 12 mL | One bottle, store 4°C |
| IMAP Reaction Buffer with Tween-20 (5x)** | 12 mL | One bottle, store 4°C |
| FAM-labeled cAMP*** Substrate | 200 µL | One vial 100 µM cAMP Substrate in 0.1% Acetic Acid + 0.01% Tween-20 Store at -20°C |
| FAM-labeled cGMP*** Substrate | 200 µL | One vial 100 µM cGMP Substrate in 0.1% Acetic Acid + 0.01% Tween-20 Store at -20°C |

* The 1X IMAP Reaction Buffer with BSA made from the supplied 5X concentrated stock contains 10 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 0.05% $NaN_3$, and 0.1% phosphate-free BSA as the carrier.
** The 1X IMAP Reaction Buffer with Tween-20 made from the supplied 5X concentrated stock contains 10 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 0.05% $NaN_3$, and 0.01% Tween-20 as the carrier.
*** TAMRA-labeled versions of these substrates are also available separately.

## Materials Required But Not Provided

| Item | Suggested Vendor |
|---|---|
| Phosphodiesterase 1 | SIGMA: P9529 |
| Calmodulin | SIGMA: P2277 |
| $CaCl_2$ | Major laboratory suppliers |
| White polystyrene 384-well microplate<br>(Corning catalog #3572 is recommended) | Major laboratory suppliers |

## Storage and Handling

Store all kit components at 4°C except the cAMP and cGMP Substrates, which should be aliquoted to avoid repeated freeze-thaw cycles and stored at -20°C.

IMPORTANT: Do Not Freeze the Binding Reagent.

When stored properly, the kit components are stable for six months from the date of receipt.

Protect substrate solutions from light.

**WARNING! Reagents can contain chemicals that are harmful. Exercise care when handling reagents as described in the related safety data sheet (SDS or MSDS). The safety data sheet is available in the Knowledge Base on the Molecular Devices support web site: support.moleculardevices.com/**

## Supported Instruments

The IMAP TR FRET Progressive Binding System is designed for use with the following instruments from Molecular Devices:

- FlexStation® 3 Multi-Mode Microplate Reader
- SpectraMax® i3x Multi-Mode Microplate Reader
- SpectraMax® iD5 Multi-Mode Microplate Reader
- SpectraMax® M4 Multi-Mode Microplate Reader
- SpectraMax® M5 Multi-Mode Microplate Reader
- SpectraMax® M5e Multi-Mode Microplate Reader
- SpectraMax® Paradigm Multi-Mode Microplate Reader

Each microplate reader has a unique set of settings to run the protocols for this reagent kit. See the *Configuring SpectraMax and FlexStation Multi-Mode Microplate Readers for IMAP Assays Setup Guide* on the Molecular Devices Knowledge Base for details.

# Chapter 3: Assay Protocols


## Quick Start Protocol

To run the Quick Start protocol:

1. 20 µL Kinase reaction/well:
   Enzyme and activators as needed.
   100 nM fluorescent labeled substrate.
2. 1 hour reaction time (or as desired).
3. Add 60 µL IMAP Binding Solution.
   FAM-cAMP:70%A, 30%B, Binding Reagent 1:800, Tb Donor: 1:400.
   FAM-cGMP:60% A, 40%B, Binding Reagent 1:800, Tb Donor: 1:400.
4. Let equilibrate 3 hours.
5. Read Tb intensity and TR-FRET.
6. Calculate corrected ratio.

Adjust volumes for plates other than standard 384-well.

## Optimization Protocol

Use this protocol as an initial guide only to detect phosphodiesterase (PDE1) activity acting on the cGMP and/or cAMP substrate. Adjust reaction concentrations, activators, and other conditions to optimize the assay system and between different PDE's. You can do this protocol at room temperature. You should keep the enzyme on ice, if possible, prior to plate addition.

Other components you can add without affecting the IMAP system are $Mn^{2+}$, $Ca^{2+}$, DTT, 2-mercaptoethanol, certain detergents, and NaCl. Some components may compete with phosphopeptide binding sites on the Binding Reagent and therefore require Binding Solution conditions different from those starting conditions listed in the *IMAP Substrates* document. These include high concentrations of EDTA and EGTA as well as phosphate and structurally related molecules.

The protocol is for 384-well plates. Adjust the volumes for 96-well or 1536-well plates.

To run the optimization protocol:

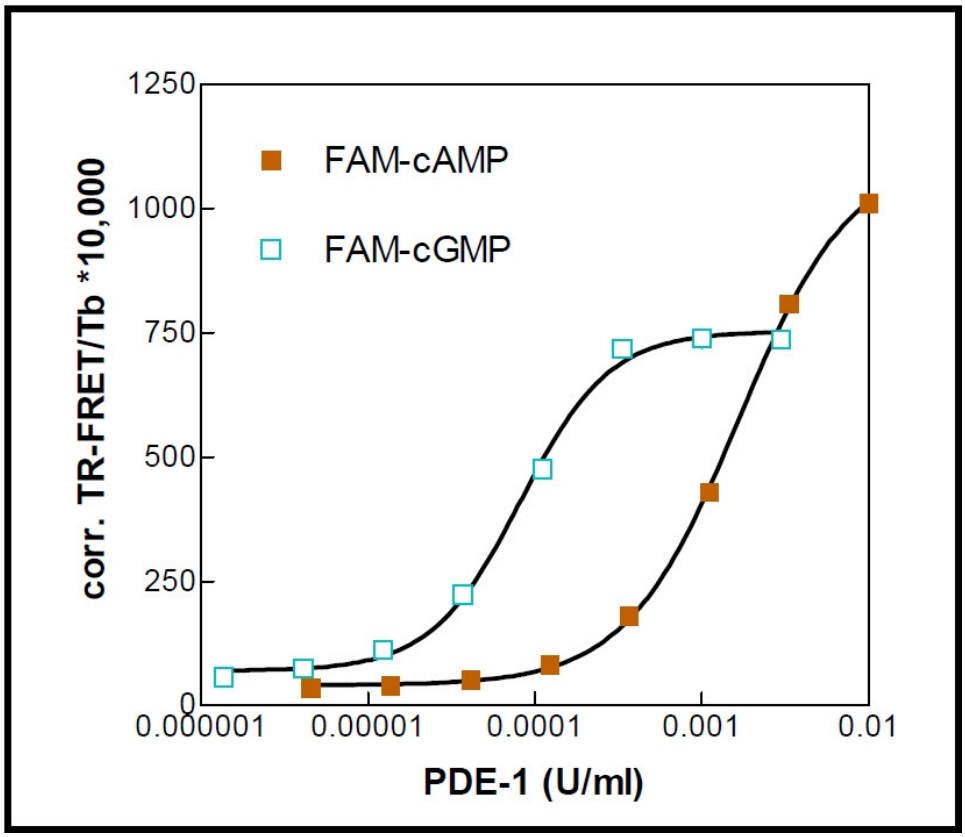
1. Prepare the Complete Reaction Buffer:
   Concerning Reaction Buffer Choice:
   IMAP Reaction Buffer with BSA provides a more sensitive assay for some PDEs in terms of $EC_{50}$, than the IMAP Reaction Buffer with Tween. However, BSA may bind to certain test compounds, possibly resulting in a rightward shift of the compound $IC_{50}$. This is a generally observed phenomenon and not IMAP-specific. If a test compound in your IMAP assay binds to BSA, you should switch the assay buffer to IMAP Reaction Buffer with Tween to overcome these issues. For evaluation purposes, both Reaction Buffer with BSA and Reaction Buffer with Tween are included in this kit.
   a. Make a 1X solution of IMAP Reaction Buffer by adding 40 mL of purified water per 10 mL of the supplied 5X concentrated stock. When stored at 4°C, the 1X solution of Reaction Buffer is stable for six months.
   b. To make the Complete Reaction Buffer, you may need to add other cofactors or DTT to a final concentration of 1 mM in the 1X solution of Reaction Buffer, depending on the requirements of the enzyme. Prepare Complete Reaction Buffer fresh for each day of assay. Approximately 15 mL is required per 384 wells. Scale up or down as needed. For the PDE1 assay as outlined below, add 1 mM DTT to make the Complete Reaction Buffer.
2. Prepare the cGMP and cAMP Substrate Working Solution:
   TR-FRET detection enables the use of flexible cAMP or cGMP concentrations. For example, to use 100 nM substrate in the reaction, add 3 µL of the 100 µM cGMP or 100 µM cAMP substrate solution per 1500 µL of Complete Reaction Buffer to make a substrate working solution (200 nM, 2x of final) for each.
3. Design the assay plate:
   a. Use the SoftMax® Pro Data Acquisition and Analysis Software to analyze the results. Set up a template before or after you read the plate. If you set up the plate before, you can print out a template to help in pipetting the plate.
   b. The following is an example of an enzyme dilution curve for PDE1 with 3 replicates:

| Column→ Row ↓ | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| A | 0.01 units/mL | 0.0033 units/mL | 0.0011 units/mL | 0.00037 units/mL | 0.00012 units/mL | 0.00004 units/mL | 0.000014 units/mL | 0 enzyme | Tb-only control | Buffer-only control |
| B | | | | | | | | | | |
| C | | | | | | | | | | |

4. Prepare the enzyme working stock solution:
   If you use PDE1 (P9529), reconstitute the lyophilized material in 50% Glycerol to 1 U/mL as recommended by the supplier. Aliquot and freeze at -20°C.
   To prepare the enzyme dilution buffer add $CaCl_2$ to 5 mM and calmodulin (Sigma) to 5,000U/mL to the IMAP Reaction Buffer prepared in step 1.
   To prepare an enzyme dilution curve as shown in step 3b, make a 180 µL stock of approximately 0.02 units/mL* in enzyme dilution buffer, and serially transfer 60 µL of this stock to 120 µL of enzyme dilution buffer. The resulting dilutions are 2x the final reaction concentrations of 0.01, 0.0033, 0.0011, 0.00037, 0.00012, 0.00004, 0.000014, and 0 units/mL.
   *One unit is defined as the amount of PDE1 (SIGMA: P9529) that will hydrolyze one µmole of cAMP per minute at 30°C and pH 7.5.
5. Add components to the 384-well assay plate:
   a. Add 10 µL of each enzyme dilution prepared in step 4 to the appropriate wells.
   b. Add 10 µL of the substrate solution prepared in step 2 to the appropriate wells.
   c. For the Buffer-Only control and the Tb-only control, add 10 µL of enzyme dilution buffer and 10 µL Complete Reaction Buffer to the appropriate wells. Each well of the assay should now have 20 µL volume.
6. Cover the plate and protect from light. Incubate at room temperature for 60 minutes. You may need to optimize reaction time for your needs.
7. Prepare sufficient IMAP Binding Solution:
   a. Make a 1X solution of Progressive Binding Buffer A and Buffer B by adding 120 mL of 5X Progressive Binding Buffer A or B to 480 mL of purified water each. When stored at 4°C, the 1X solutions of Progressive Binding Buffer A or B are stable for 6 months.
   b. Reconstitute the Tb-Donor by adding 0.15 mL water to the lyophilized Tb Donor vial and mix well. Store at 4°C.
   c. The optimal Binding Buffer composition for FAM-cAMP is: 70% Binding Buffer A, 30% Binding Buffer B and Binding Reagent 1:800, Tb Donor 1:400. For example, to make 20 mL of this Binding Solution combine: 14 mL 1x Binding Buffer A, 6 mL 1x Binding Buffer B, mix thoroughly; add 25 µL Progressive Binding Reagent, mix, add 50µL Tb-Donor and mix again. For the Buffer-only control, set aside a small amount of Binding Solution without Tb Donor.
   d. The optimal Binding Buffer composition for FAM-cGMP is: 60% Binding Buffer A, 40% Binding Buffer B and Binding Reagent 1:800, Tb Donor 1:400. For example, to make 20 mL of this Binding Solution combine: 12 mL 1x Binding Buffer A, 8 mL 1x Binding Buffer B, mix thoroughly; add 25 µL Progressive Binding Reagent, mix; add 50 µL Tb-Donor and mix again. For the Buffer-only control, set aside a small amount of Binding Solution without Tb Donor.
   e. Add 60 µL of Binding Solution with Tb Donor to each assay well, including the Tb- only control wells. Add 60 µL of Binding Solution without Tb Donor to the Buffer-only control wells.
8. Cover the plate and protect from light. Incubate at room temperature for 3 hours. Longer incubation times can provide a slight increase in response.
9. The SoftMax Pro Software settings to read the plate depend upon the microplate reader you use. See the *Configuring SpectraMax and FlexStation Multi-Mode Microplate Readers for IMAP Assays Setup Guide* document for details.

10. Analyze the results:
    (exemplified for FAM wavelengths, for TAMRA change to appropriate wavelengths)
    To correct for Tb donor contribution to the FRET signal, first the proportionality factor P is calculated:
    P = Tb520/Tb490
    Where Tb520 = average RFU at 520 nm in the Terbium-only control minus average RFU at 520 nm in the Buffer-only control and Tb490 = RFU at 490 nm in the Terbium-only control minus RFU at 490 nm in the Buffer-only control
    P is used in the calculation of the corrected FRET ratio:
    Corrected FRET ratio = [(FRETsample-(P*Sample490))/Sample490]*10000
    Where FRETsample = RFU at 520 nm in one assay well minus average RFU at 520 nm in the Buffer-only control, and Sample 490 = RFU at 490 nm in one assay well minus average RFU at 490 nm in the Buffer-only control.

# Chapter 4: Sample Data




Titration of PDE1 with the IMAP TR-FRET Phosphodiesterase Assay Kit in the presence of 2.5 mM $CaCl_2$ and 2500 U/mL calmodulin

# Obtaining Support

Molecular Devices is a leading worldwide manufacturer and distributor of analytical instrumentation, software, and reagents. We are committed to the quality of our products and to fully supporting our customers with the highest level of technical service.

Our Support website—support.moleculardevices.com/—describes the support options offered by Molecular Devices, including service plans and professional services. It also has a link to the Molecular Devices Knowledge Base, which contains documentation, technical notes, software upgrades, safety data sheets, and other resources. If you still need assistance, you can submit a request to Molecular Devices Technical Support.

Please have the instrument serial number (on the rear of the instrument), or reagent kit lot number, and any related sample data files available when you call.

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