IMAP TR-FRET Progressive Binding Systems

The IMAP® TR-FRET Progressive Binding System antibody-free, time-resolved fluorescence resonance energy transfer (TR-FRET) assay kit, called IMAP TR-FRET, combines all the advantages of IMAP (an antibody-free, stable, generic, homogeneous format) with the advantages of TR-FRET for detection of phosphorylation. These advantages include: reduced minimum percentage substrate conversion, increased substrate size and concentration flexibility, and low background due to time-resolved detection. You can use IMAP TR-FRET in conjunction with fluorescent substrates to evaluate enzyme activity of kinases. The assay is a simple, homogeneous mix and read procedure that allows accurate determination of enzyme activity.

Available Kits

	Data Points	Kit
IMAP TR-FRET Evaluation Kit	800	R8161
IMAP TR-FRET Screening Express Kit	8,000	R8160

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Chapter 1: About the IMAP TR-FRET Progressive Binding Systems

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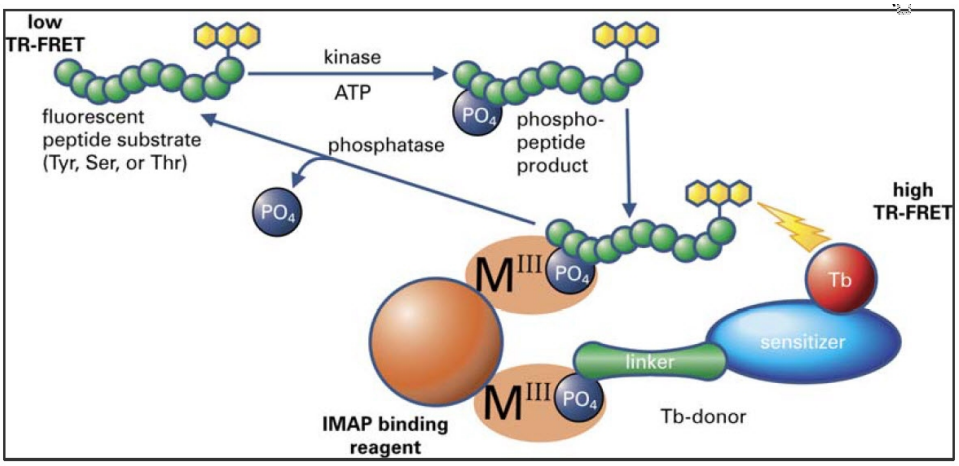
The Progressive Binding System is offered as a mix-to-match system, which allows you to optimize your assay to achieve the best possible results for each application in a fast and simple manner. Examine the ratio of Progressive Buffers A and B during assay development to determine the optimal conditions for the assay of interest. See Optimization Protocol on page 7.

Assay Principle

The IMAP TR-FRET System utilizes all components of the IMAP FP system as well as an IMAP Terbium (Tb) labeled TR-FRET donor that consists of a phosphate-containing linker, a sensitizer and a Tb complex combined in one molecule. The Binding Solution in the IMAP FP platform consists of IMAP Binding Buffer that contains IMAP Binding Reagent. In IMAP TR-FRET, a similar Binding Solution is used to which the Tb Donor is added. The Donor binds to the IMAP binding entities. Upon phosphorylation by a kinase, the fluorescent phosphopeptide product of the kinase reaction is brought in close proximity to the Tb that contains Donor by also binding to the IMAP binding entities, thus producing resonance energy transfer. Due to the long lifetime of Tb fluorescence, you can run the detection in time-resolved mode, which virtually eliminates fluorescence interference from assay components or compounds in a screen. This assay is applicable to a wide variety of kinases without regard to the substrate peptide sequences and is antibody independent.



Principle of the IMAP TR FRET System

Applications

The IMAP Kits are designed for use in biochemical assays of kinase, phosphatase, and phosphodiesterase activity. They are especially suited for high throughput screening applications. Assays for kinases from all areas of the kinome have been successfully developed.

Chapter 2: Materials and Equipment

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Kit Components

Components of the Assay Kits

	IMAP TR-FRET Evaluation Kit (R8161)	IMAP TR-FRET Screening Express (R8160)	Description
Assay Kit Capacity	800 data points	8,000 data points	Assay kits contain sufficient IMAP reagents to generate 800 to 8,000 data points for the Quick Start protocol. See Quick Start Protocol on page 7.
IMAP Progressive Binding Reagent	0.15 mL	1.5 mL	One vial, store 4°C **Do not freeze the Binding Reagent**
IMAP Progressive Binding Buffer A (5x)	12 mL	120 mL	One bottle, store 4°C
IMAP Progressive Binding Buffer B (5x)	12 mL	120 mL	One bottle, store 4°C
TR-FRET Donor	lyophilized	lyophilized	One vial, store 4°C
IMAP Reaction Buffer with BSA (5X)*	12 mL	120 mL	One bottle, store 4°C The Complete Reaction Buffer consists of a 1X stock with added DTT (1 mM final reaction concentration).
IMAP Reaction Buffer with Tween-20 (5X)**	12 mL	120 mL	One bottle, store 4°C The Complete Reaction Buffer consists of a 1X stock with added DTT (1 mM final reaction concentration).

* 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.

Materials Required But Not Provided

Item	Suggested Vendor
White polystyrene 384-well microplate (Corning catalog #3572 is recommended)	Major laboratory suppliers (MLS)
Adenosine 5' Triphosphate (ATP), 10 mM stock in purified water, aliquots at -70°C recommended	MLS
DL-Dithiothreitol (DTT), 100 mM stock in purified water, aliquots at -20°C recommended	MLS
Kinase, phosphatase, or Phosphodiesterase enzyme	Cell Signaling Technologies, Upstate Biotechnology or other source
Fluorescent peptide substrate	See *IMAP Substrates* document

Storage and Handling

Store all kit components at 4°C.

IMPORTANT: Do Not Freeze the Binding Reagent.

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

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:
   Kinase as needed.
   Fluorescently labeled substrate as desired (commonly tested 0.3 - 3 µM).
   100 µM ATP (or as desired) in complete IMAP Reaction Buffer.
2. 1 hour reaction time (or as desired).
3. Add 60 µL IMAP Binding Solution. See Recommended Initial Binding Buffer Conditions on page 12.
4. Let equilibrate according to Binding Buffer composition.
5. Read TR-FRET.

Adjust volumes for plates other than standard 384-well.

Optimization Protocol

Use this protocol as an initial guide only. Adjust buffer concentrations and other conditions to optimize the assay system. You can do this protocol at room temperature. You should keep the enzyme on ice, if possible, prior to adding it to the assay plate.

Other components you can add without affecting the IMAP system are 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 the starting conditions. These include high concentrations of EDTA and EGTA as well as phosphate and structurally related molecules.

IMAP TR-FRET Progressive Binding System Components

Reagent	Description
IMAP Progressive Binding Buffer A	Baseline binding buffer.
IMAP Progressive Binding Buffer B	Affects TR-FRET background by reducing, or blocking, the non-phosphatebased 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 fluorophor on phosphorylated substrate by binding both even under very stringent Binding Buffer conditions to the Binding entities.

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

To run the optimization protocol:

1. The key to smooth development of an IMAP screen with the Progressive Binding System lies in taking a few steps to fine-tune the assay response:
   Count the number of carboxyl groups (-COOH) in the peptide. If the peptide is a free acid rather than amidated on the C-terminus, be sure to include the terminal -COOH in your count. Beyond depending on the number of acidic residues, the optimal Binding Buffer condition also depends on the total hydrophobicity of the peptide and the number of residues that contain Hydroxy groups (Ser, Thr, Tyr). Both parameters tend to increase the need for more Buffer B relative to the conditions stated (see Recommended Initial Binding Buffer Conditions on page 12), which are averages and were found to work well for the validated IMAP substrates.
2. The starting Progressive Binding Buffer formulation, as optimized for TR-FRET detection for 300 nM –3 µM substrate in IMAP reaction buffer with 0.01% Tween and 1 mM DTT in the presence of 100 µM ATP for all peptide substrates available from Molecular Devices, is listed in the *IMAP Substrates* document included with this kit. For Tyrosine Kinase substrates, the reaction buffer also contained 2 mM $MnCl_2$ during optimization.
   The same document also contains the optimal starting conditions for FP detection using the IMAP FP Buffer kits.
   a. If your peptide is not listed, see Recommended Initial Binding Buffer Conditions on page 12 to determine the starting Binding Solution conditions depending on the number of carboxyl groups in the peptide.
   b. The parameters you should consider for Binding Solution Optimization are: TR-FRET-signal increase normalized to Tb intensity (ratio) and Z-factors at the approximate % substrate phosphorylation you target.

3. To test the assay system, prepare several IMAP Binding Solutions with different compositions that center around the general specifications in the Recommended Initial Binding Buffer Conditions on page 12 as a guide.
   a. Make a 1X solution of Progressive Binding Buffer A and Buffer B by diluting both 1:5 (for example, add 120 mL of 5X Progressive Binding Buffer to 480 mL of purified water). 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 water as indicated in the following table to the lyophilized Tb Donor vial and mix well. Store at 4° C.

Kit Type (part number)	Data Points	Reconstitution Volume
Evaluation kit (R8161)	800	0.15 mL
Screening Express (R8160)	8,000	1.5 mL

   c. Procedure for an example IMAP TR-FRET Binding Solution:
      - Mix 1x IMAP Binding Buffers A and B at desired ratio.
      - Add IMAP Binding reagent at desired dilution and mix.
      - Add Tb Donor 1:400 and mix.

      For example, to make 20 mL Binding Solution that uses 80% Binding Buffer A, 20% Binding Buffer B, 1:600 Binding Reagent, and Tb donor at 1:400, first combine 16 mL 1x Progressive Binding Buffer A and 4 mL 1x Progressive Binding Buffer B. Mix. Add 33.3 µL of Progressive Binding Reagent. Mix. Then add 50 µL of the Tb Donor. Be careful to pipet the Progressive Binding Reagent accurately. Assay-to-assay variation can occur if you pipet very small volumes of the Progressive Binding Reagent each time (< 20 µL). The Binding Solution should be freshly prepared each day of assay.

Note: To make an intermediate dilution of the Progressive Binding Reagent, use 0.1 N HCl. This intermediate dilution should be at least 100X of the final working dilution and should not be stored and reused. Do not use Binding Buffers to make this intermediate dilution.

4. Prepare the Complete Reaction Buffer.
   Concerning Reaction Buffer Choice:

   Note: You can use either the IMAP Reaction Buffer that contains 0.1% BSA or the Reaction Buffer that contains 0.01% Tween-20 in the IMAP TR-FRET assay. This example protocol only mentions the Reaction Buffer with Tween-20.

   Reaction Buffer with BSA often provides a more sensitive assay, in terms of enzyme EC_{50}, than the 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 TR-FRET- or FP-specific.
   If either a test compound or substrate in the IMAP assay binds to BSA, you should switch the assay buffer to Reaction Buffer with Tween to overcome these issues. In some cases, assays that utilize buffer with BSA yielded a lower background. This background behavior is highly specific for each peptide sequence. The Binding Buffer conditions described for IMAP TR-FRET as the starting point for assay development in the *IMAP Substrates* document are optimized for 0.3 - 3 µM of that substrate in Reaction Buffer with Tween (with 1 mM DTT) in the presence of 100 µM ATP, unless stated otherwise. If you use different reaction conditions, you should do a quick background check of the substrate in those assay conditions. For evaluation purposes, both Reaction Buffer with BSA and Reaction Buffer with Tween are included in the 800 data point and 8,000 data point kits.
   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 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.
5. Prepare a 1x solution of the Substrate in Complete IMAP Reaction Buffer (or the reaction buffer you will use in the assay).
   a. Assay optimization with substrate and phosphorylated substrate (Calibrator) on hand:
   Make a 1x solution of both peptides in Complete Reaction Buffer that contains the concentration of ATP you plan for the assay, if applicable. Mix substrate and calibrator solutions at different ratios to imitate different % phosphorylation. Normal focus is on lower % phosphorylation (5% to 30%). Pipet 20 µL of these peptide solutions into a 384-well white plate as a small standard curve. Add 60 µL of the Progressive Binding Solutions made in step 3 to each curve. Cover the plate and incubate at room temperature for the time listed in Recommended Initial Binding Buffer Conditions on page 12 or the *IMAP Substrates* document. Measure the TR-FRET.
   b. Assay optimization without Calibrator (with enzyme):
   Run an enzyme dilution curve that uses 100 µM ATP, 0.3 or 1 µM FAM or TAMRA labeled substrate in 20 µL reaction buffer. Add 60 µL Binding Solution. Choose this Binding Solution composition according to Recommended Initial Binding Buffer Conditions on page 12 based on the number of acidic residues in the substrate.
   Choose an enzyme concentration around the EC_{50} of the resulting curve. Run with no enzyme and one fixed enzyme concentration in the presence of ATP and substrate concentration. Combine with several Binding Solutions revolving around the one recommended in Recommended Initial Binding Buffer Conditions on page 12 as described in step 3.

6. The SoftMax® Pro Data Acquisition and Analysis 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.
7. Analyze the data:
   a. It is important to take into account the Background Fluorescence of the assay and the crosstalk (Tb intensity as read in FAM or TAMRA channel). These parameters may vary for different instrument or assay plates.
      For a very detailed publication on background and crosstalk correction see: Gordon et al. (1998): Biophys. J. 71, 2702-2713.
   b. Suggested parameters to choose the optimum Binding Solution are: TR-FRET-signal increase normalized to Tb intensity (ratio) and Z-factors at the approximate % substrate phosphorylation targeted.
   c. If the assay contains significant amounts of other components, such as EDTA, EGTA, phosphate, or phosphate-like molecules such as sodium orthovanadate, you may need to increase the concentration of the Progressive Binding Reagent in the assay to increase the phosphate binding capacity.

Recommended Initial Binding Buffer Conditions

The Binding Buffer Conditions described in the *IMAP Substrates* document were determined for substrates at 100 nM in IMAP Reaction Buffer with Tween (with 1 mM DTT) in the presence of 100 µM ATP (unless stated otherwise).

Starting Conditions for Optimization of Progressive Binding Solutions for TR-FRET Detection

Number of -COOHs in peptide	Progressive Binding Buffer A	Progressive Binding Buffer B	Progressive Binding Reagent Dilution	Binding Incubation time (minimum)
None	80%	20%	1/600	30 minutes
1	70%	30%	1/600	2 hours
2	40%	60%	1/800	4 hours
3	30%	70%	1/800	5 hours or more*
4	0%	100%	1/1000	5 hours or more*
5 or more	0%	140%**	1/1000	5 hours or more*

* depending on number of acidic residues in peptide.

** For peptide substrates that require greater than 100% 1x Buffer B, you must use a >1x stock, for example, 160% 1x Buffer B = 1.6x Buffer B. Using the 5x stock, make a 1: 3.13 dilution.

Chapter 4: Sample Data

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With IMAP TR-FRET Progressive Binding System, you can achieve more flexibility with IMAP for low affinity, acidic peptide sequences and higher ATP concentrations and you gain more freedom in substrate concentration and more robust assays at lower % phosphorylation.

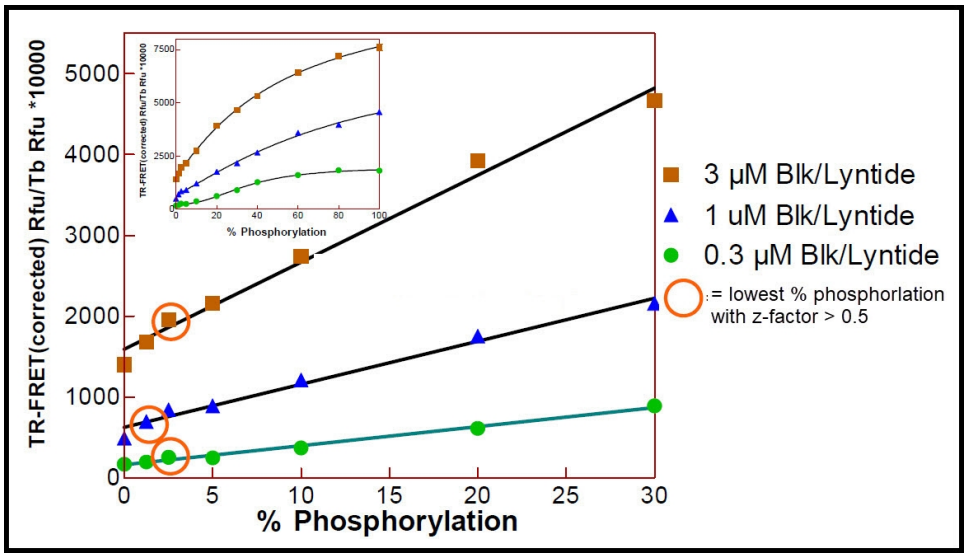

Figure 2. IMAP TR-FRET Calibration curve with FAM- Blk/Lyntide (5FAM-EFPIYDFLPAKKK-NH_2)

Non-phosphorylated and phosphorylated Blk/Lyntide at concentrations as indicated were mixed to create the percentage phosphorylation levels. Solutions were made in complete IMAP reaction buffer in the presence of 100 µM ATP. Twenty µL peptide solution was pipetted per well. 60 µL of Binding Solutions (30% Binding Buffer A, 70% Binding Buffer B, Binding Reagent 1:800 and Tb Donor 1:400) were added. TR-FRET was read after 3 hours. The red circles indicate the lowest % phosphorylation at which the z-factor surpasses 0.5.

Figure 2 shows the example of Blk/Lyntide. With IMAP TR-FRET, a % Phosphorylation below 5% generates a z-factor greater than 0.5. This represents a significant improvement in sensitivity over IMAP FP, which requires a %phosphorylation greater than 20% to achieve a value of 0.5 for the z-factor.

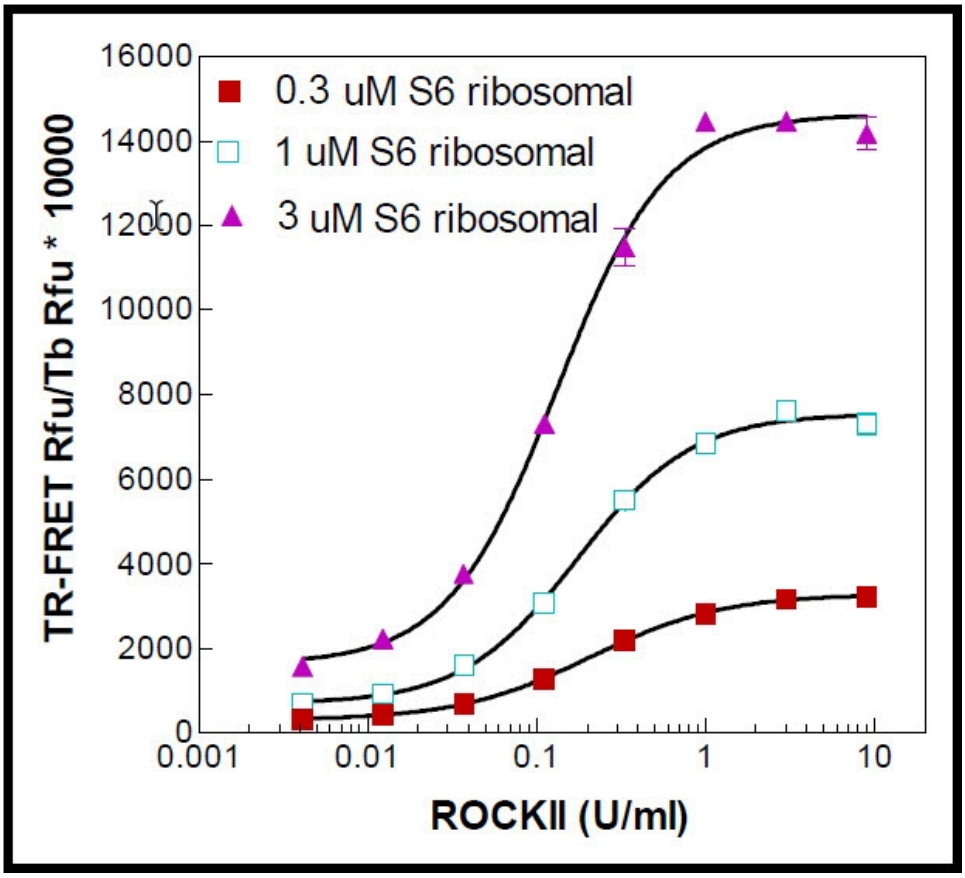

Figure 3. IMAP TR-FRET ROCKII assay using the IMAP reaction buffer with Tween (incl. 1mM DTT)

Enzyme (Upstate cat# 14-451) was incubated with S6 derived substrate (5FAM-AKRRRLSSLRA-COOH, R7184) at the concentrations indicated in the presence of 100 µM ATP for 1 hour in 20 µL reaction volume. 60 µL Progressive Binding Solution was added (70% Binding Buffer A, 30% Binding Buffer B, 1:600 dilution of Progressive Binding Reagent and Tb-Donor diluted 1:400). The TR-FRET was read after 2 hours incubation following addition of the Binding Solution.

Figure 3 shows an IMAP TR-FRET enzyme dilution curve for ROCKII using 0.3 - 3 µM FAM-labeled substrate. The data show the assay window, sensitivity and response to different substrate concentrations. Use the change of response as a function of substrate concentration to determine substrate EC_{50} directly as shown in Figure 4. You can use this data to determine the Km.

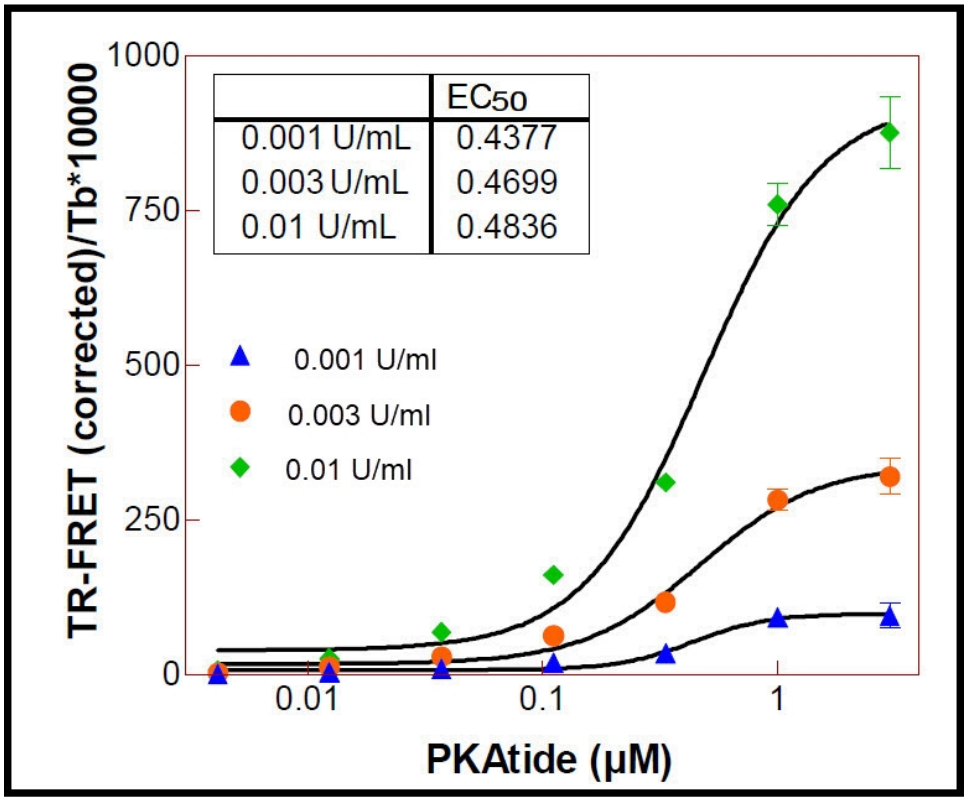

Figure 4. Substrate dependency for FAM-PKAtide with PKA using IMAP reaction buffer with Tween (incl 1 mM DTT).

Reaction conditions: PKA kinase (Upstate: 14-440) was incubated with increasing concentrations of FAM-PKAtide as indicated (5FAM-GRTGRRNSI-NH_2, R7250), in the presence of 100 µM ATP for 1 hour at room temperature.

IMAP Binding Solution was added (95% Binding Buffer A, 5% Binding Buffer B, Progressive Binding Reagent 1:400, Tb Donor 1:400). TR-FRET was read 2 hours after Binding Solution addition.

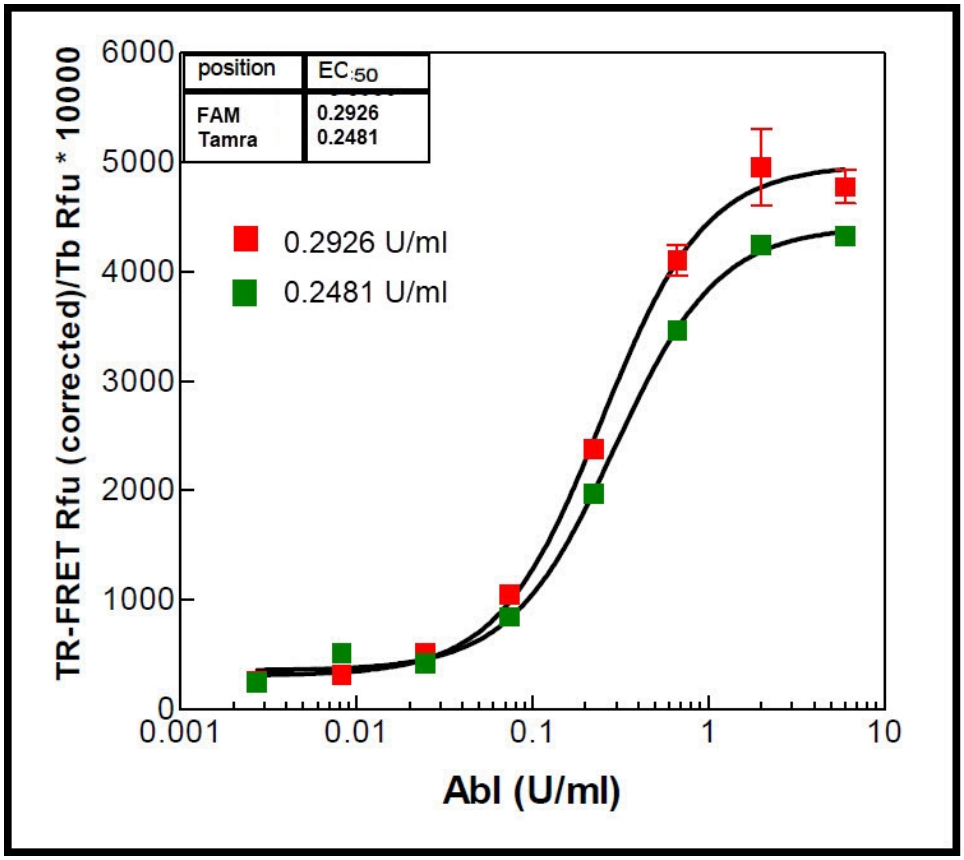

Figure 5. IMAP TR-FRET assay for Abl using TAMRA and FAM labeled Abltide (FL-KKGEAIYAAPFA-NH_2) in Tween reaction buffer (incl. 1mM DTT).

The enzyme (Upstate #14-529) was titrated in the presence of 100 µM ATP and 1 µM substrate in a 1 hour reaction. Binding Solution was added (80% Binding Buffer A, 20% Binding Buffer B, Binding Reagent 1:800, Tb Donor 1:400). TR-FRET was read after 4 hours.

Figure 5 shows the suitability for IMAP TR-FRET for TAMRA and FAM labeled substrates.

About Substrates

The IMAP TR-FRET Binding System Kits are designed to provide flexibility in designing peptide substrates. Molecular Devices offers a variety of lyophilized peptide substrates that work with the IMAP system (FP and TR-FRET detection). See the *IMAP Substrates* document.

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), and any related sample data files available when you call.

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