IMAP FP Progressive Binding Systems

The IMAP® FP Progressive Binding Systems IMAP Buffer Kits, based on IMAP's antibody-free detection of phosphorylation, can be used 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 configured to allow you to determine maximum performance for every possible application. The IMAP Progressive Binding System kits described in this product insert are designed for detection in Fluorescence Polarization mode.

Available Kits

	Data Points	Kit
IMAP FP Evaluation Kit	800	R8155
IMAP FP Screening Express Kit	8,000	R8127
IMAP FP IPP Explorer Kit	8,000	R8124

Contents

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MOLECULAR DEVICES

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Chapter 1: About the IMAP FP Progressive Binding Systems

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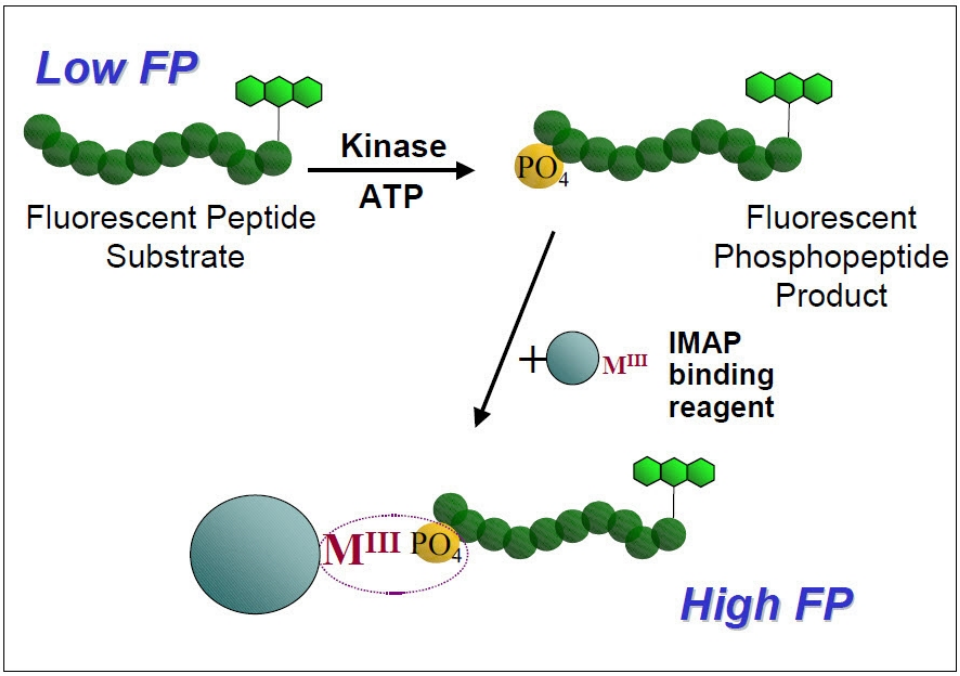
The Progressive Binding System is offered as a mix-to-match system, which allows you to optimize your assay to achieve the best possible results for each application in a fast and simple manner. Examine the ratio of Progressive Buffers A and B during assay development to determine the optimal conditions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

Assay Principle

The IMAP 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. Such binding causes a change in the rate of the molecular motion of the peptide, and results in an increase in the fluorescence polarization value observed for the fluorescent label attached at the end of the peptide. This assay, unlike antibody-based kinase assays, is applicable to a wide variety of kinases without regard to the substrate peptide sequences.



Principle of the IMAP FP 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

Kit Components

Components of the IMAP FP Progressive Binding System

	IMAP FP Evaluation Kit (R8155)	IMAP FP Screening Express (R8127)	IMAP FP IPP Explorer (R8124)*	
Assay Kit Capacity	800 data points	8,000 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	1.5 mL	One vial, store 4°C **Do not freeze the Binding Reagent**
IMAP Progressive Binding Buffer A (5x)	12 mL	120 mL	120 mL	One bottle, store 4°C
IMAP Progressive Binding Buffer B (5x)	12 mL	120 mL	120 mL	One bottle, store 4°C
IMAP Reaction Buffer with 0.1% BSA (5X)**	12 mL	120 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 0.01% Tween-20 (5X)***	12 mL	120 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).

* R8124 is available only for IPP (IMAP Purchase Program) members with an annual subscription fee.

** The 1X IMAP Reaction Buffer, 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, 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
Black polystyrene 384-well microplate (Corning catalog #3573 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 included *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 FP Progressive Binding System is designed for use with the following instruments from Molecular Devices:

- SpectraMax® i3x Multi-Mode Microplate Reader
- SpectraMax® iD5 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.
   - Fluorescent 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 10.
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 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. These include high concentrations of EDTA and EGTA as well as phosphate and structurally related molecules.

IMAP FP Progressive Binding System Components

Reagent	Description
IMAP Progressive Binding Buffer A	Baseline binding buffer
IMAP Progressive Binding Buffer B	Affects FP 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.

To run the standard 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.
2. The starting Progressive Binding Buffer formulation as optimized for FP detection for 100 nM substrate in IMAP reaction buffer with BSA 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.
   The same document also contains the optimal starting conditions for TR-FRET detection using the IMAP TR-FRET Buffer kits.
   a. If your peptide is not listed, see Recommended Initial Binding Buffer Conditions on page 10 to determine the starting Binding Solution conditions depending on the number of carboxyl groups in the peptide.
   b. If you develop a phosphatase assay, you must use the non-phosphorylated form of the peptide to perform the optimization steps. If only the phosphorylated substrate is available, you need to perform the phosphatase reaction first to dephosphorylate the peptide to optimize the binding system.
3. Prepare 10 mL of Binding Solution according to the general specifications in Recommended Initial Binding Buffer Conditions on page 10 or as specified in the *IMAP Substrates* document.
   a. Make a 1X solution of Progressive Binding Buffer A and Buffer B by adding 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. For example, if the peptide contains 3 carboxyl groups, add together 7.5 mL 1X Progressive Binding Buffer A, 2.5 mL 1X Progressive Binding Buffer B and 16.7 µL of Progressive Binding Reagent. Be careful to pipet the Progressive Binding Reagent accurately. Assay-to-assay variation can occur if very small volumes of the Progressive Binding Reagent are pipetted each time. 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:
   IMAP Reaction Buffer with BSA provides a more sensitive assay, 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. Peptide substrates that contain a number of hydrophobic residues have also been known to bind BSA in IMAP assays, resulting in an elevated peptide FP background.
   If either a test compound or substrate in the IMAP assay binds to BSA, you should switch the assay buffer to IMAP Reaction Buffer with Tween to overcome these issues. In some cases, assays that use buffer with BSA yielded a lower background. This background behavior is highly specific for each peptide sequence. The Binding Buffer conditions described as the starting point for assay development in the *IMAP Substrates* document are optimized for 100 nM of that substrate in IMAP Reaction Buffer with BSA (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 100 nM solution of the peptide in Complete IMAP Reaction Buffer (or the reaction buffer you use in the assay). Make a 100 nM solution of the peptide in Complete Reaction Buffer that contains the concentration of ATP planned for the assay, if applicable. Pipet 20 µL of this peptide solution into a 384-well black plate and add 60 µL of the Progressive Binding Solution you made in step 3. Cover the plate and incubate at room temperature for the time listed in Recommended Initial Binding Buffer Conditions on page 10 or the *IMAP Substrates* document. Measure the fluorescence polarization (FP).
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. If the background (non-phosphorylated peptide only) FP is acceptable (usually less than 150 mP), further fine-tune the assay to allow for very high ATP concentrations (up to 500-1000 µM ATP, see Figure 4). To accomplish this, increase the concentration of the Progressive Binding Reagent in the Binding Solution and then recheck the background FP. If the assay contains 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.
   b. To lower the background FP, increase the amount of Progressive Buffer B in the Binding Solution and recheck the background FP.

Recommended Initial Binding Buffer Conditions

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

Starting Conditions for Optimization of Progressive Binding Solutions for FP Detection

Number of -COOHs in Peptide	Progressive Binding Buffer A	Progressive Binding Buffer B	Progressive Binding Reagent Dilution	Binding Incubation Time (minimum)
None or 1	100%	0%	1/400	30 minutes
2 or 3	75%	25%	1/600	1 hour
4	40%	60%	1/1200	2 hours
5 or more	25%	75%	1/2500	5 hours or more, depending on number of acidic residues in peptide

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You can achieve good results under challenging conditions such as in assays that use acidic peptide sequences or high ATP concentrations with the Progressive Binding System. Figure 2 shows the titration of the kinase CDK2/Cyclin A. This substrate is not acidic, so a simple 100% A binding solution is used.

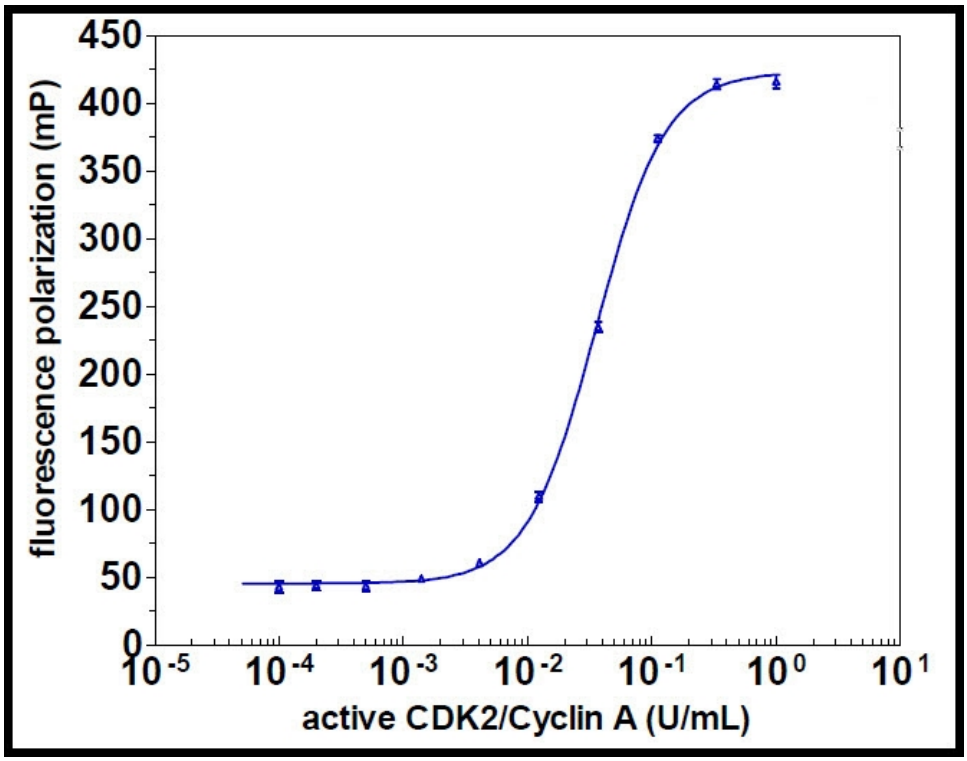

Figure 2. IMAP CDK2/Cyclin A enzyme dilution curve using the IMAP reaction buffer with BSA.

The enzyme (Upstate cat #14-458) was titrated in the presence of 30 µM ATP and 100 nM FAM-Histone H1-derived peptide in a 1 hour reaction. Progressive Binding Solution of 100% Buffer A and 1/400 dilution of Progressive Binding Reagent was used. The FP was read after 1 hour incubation following addition of the Binding Solution.

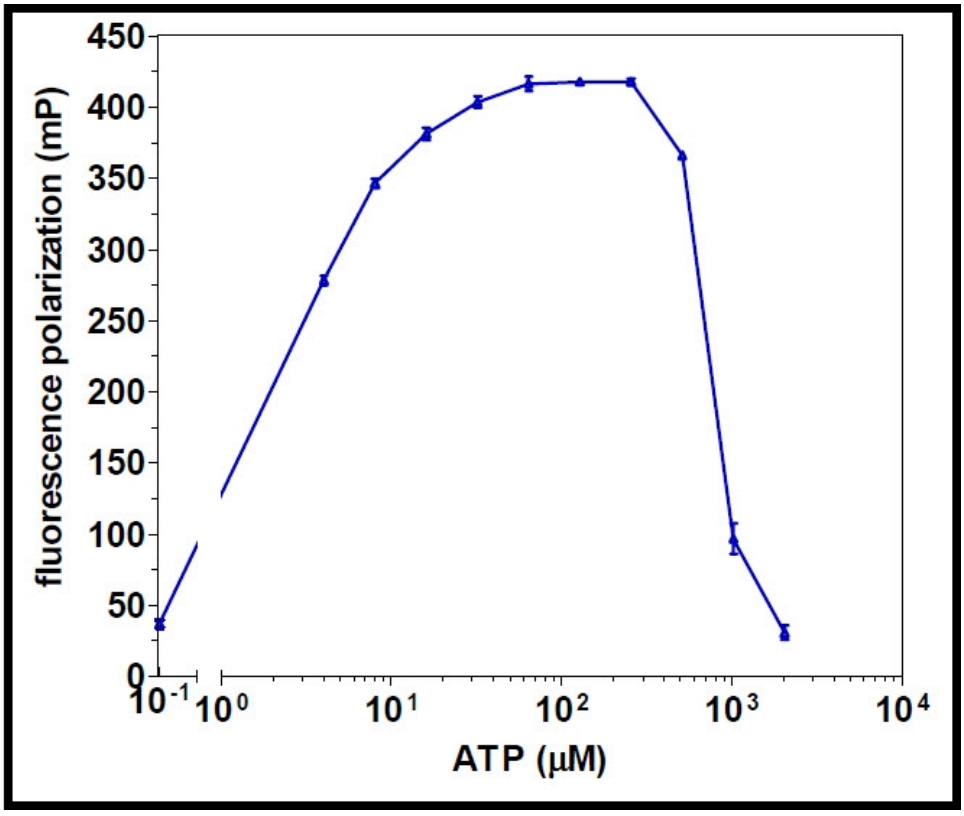

Figure 3. IMAP CDK2/Cyclin A assay using the IMAP reaction buffer with BSA.

ATP was titrated with an EC_{70} concentration of CDK2/Cyclin A enzyme and 100 nM FAM-Histone H1-derived peptide in a 1 hour reaction. Progressive Binding Solution of 100% Buffer A and 1/400 dilution of Progressive Binding Reagent was used. The FP was read after 1 hour incubation following addition of the Binding Solution.

This data shows the binding capacity with the Progressive System tolerates ATP concentrations up to 200 µM. This is sufficient to determine the K_m for ATP and to run assays above the K_m of ATP, even for a kinase such as CDK2/Cyclin A that has a relatively high K_m value (~80 µM). To accommodate significantly higher ATP concentrations, adjust the Binding Buffer composition, as shown in Figure 4. In this experiment, dilution curves with Fyn tyrosine kinase were run at 30 µM ATP and 1000 µM ATP using the TAMRA-labeled p34cdc2-derived peptide as the substrate. Good assay windows were achieved through an increase of Binding Reagent in the Binding Solution.

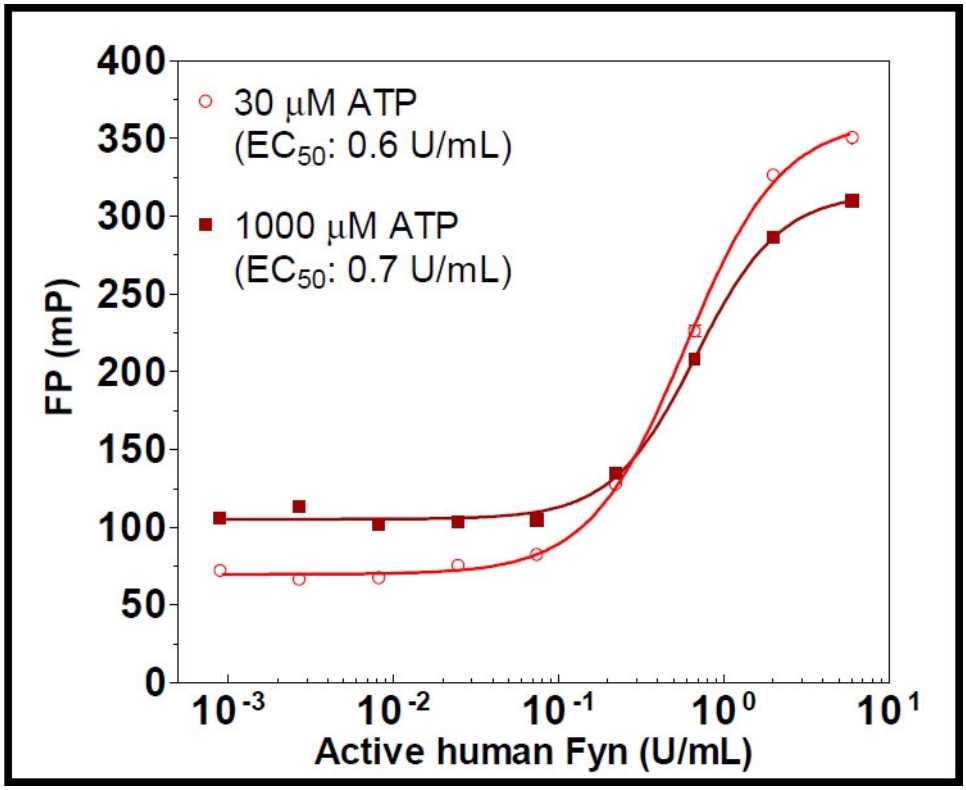

Figure 4

IMAP Fyn dilution curve using IMAP reaction buffer with BSA, 2 mM $MnCl_2$, 100 nM TAMRA-p34cdc2-derived peptide.

The enzyme (Upstate cat #14-441) was titrated in the presence of 30 µM ATP (open circles) and 1 mM ATP (solid squares). For samples that contain 30 µM ATP, Binding Solution of 75% Binding Buffer A, 25% Binding Buffer B, and 1/600 dilution of Progressive Binding Reagent was used. To accommodate 1 mM ATP the amount of Binding Reagent was increased to a dilution of 1/300. FP was read after a 2 hour incubation following addition of the Binding Solution.

The Progressive Binding System allows you to use acidic peptides in IMAP. CK2tide (FAM-RRRADDSDDDDD- NH_2) is an extremely acidic substrate, which requires 12% Binding Buffer A, 79% Binding Buffer B, 9% 5M NaCl, and Binding Reagent 1:2500 as the Binding Solution. Even under these extreme buffer conditions, you can achieve a good assay window with IMAP. For this kind of high acidic content (7 aspartates out of 12 amino acids total), an overnight incubation after Binding Solution addition is recommended to allow for complete signal stabilization.

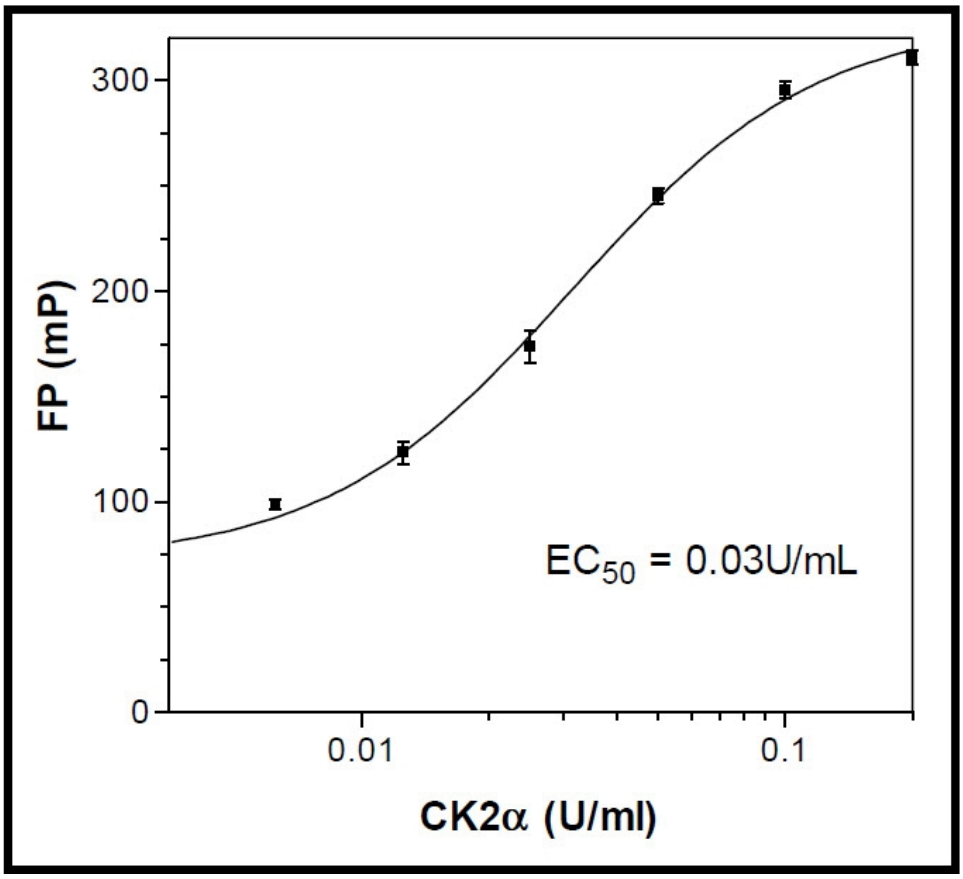

Figure 5. IMAP CK2α assay using IMAP reaction buffer with BSA.

The enzyme (Upstate #14-445) was titrated in the presence of 10 µM ATP and 100 nM CK2tide in a 1 hour reaction. Binding Solution of 12% Binding Buffer A, 79% Binding Buffer B, 9% 5M NaCl and a dilution of 1/2500 Binding Reagent was added. The FP was read following overnight incubation.

Included in the IMAP Progressive Binding System kits are two Reaction Buffers, one uses 0.01% Tween-20 and one uses 0.1% BSA as carriers. Figure 6 shows dilution curves for the calmodulin and Ca^{2+}-activated phosphatase Calcineurin in Reaction Buffer with Tween as compared to Reaction Buffer with BSA. It shows that, in this case, Reaction Buffer with Tween slightly increases the sensitivity of the assay. You may need to evaluate additional parameters when you choose the reaction buffer. See Recommended Initial Binding Buffer Conditions on page 10.

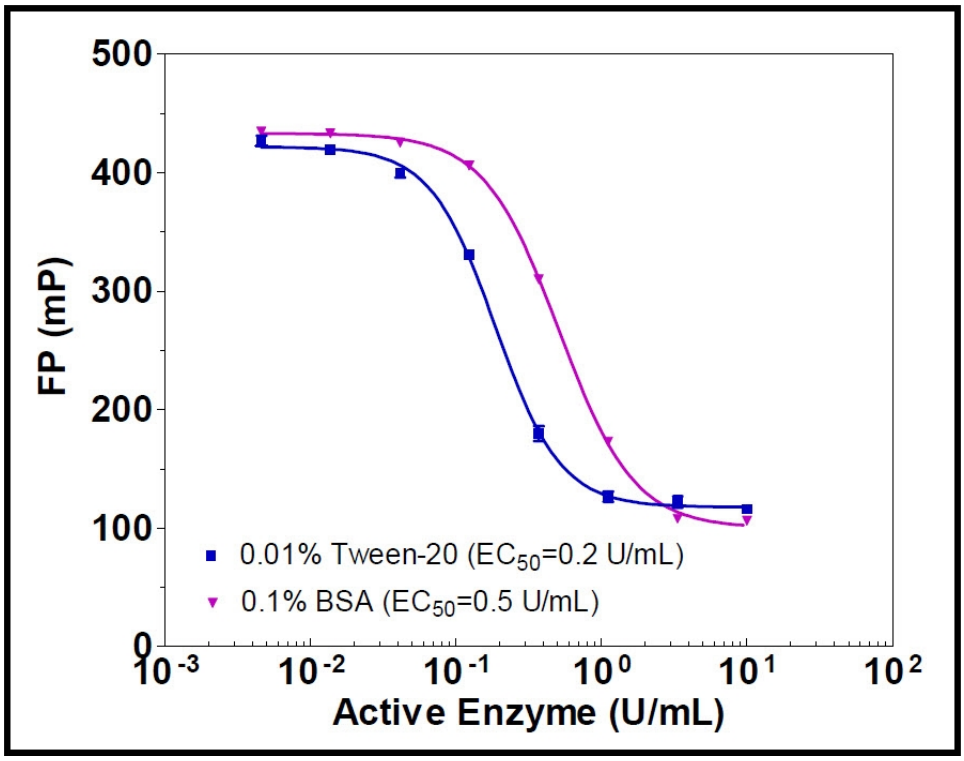

Figure 6. IMAP Calcineurin assay using the IMAP Reaction Buffer with BSA or with Tween, 1 µM calmodulin (Upstate 14-368) and 100 µM Ca^{2+}.

The enzyme (Upstate cat #14-446) was titrated using 100 nM PKA regulatory subunit-derived phosphopeptide. Binding Solution was comprised of 75% Binding Buffer A, 25% Binding Buffer B and a dilution of 1/600 Progressive Binding Reagent.

About Substrates

The IMAP FP Progressive 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. See the *IMAP Substrates* document or contact Molecular Devices technical support for more information.

You can design your own fluorescent peptide substrates. You should label substrates with fluorescein or other FP competent fluorophores like rhodamine 6G, TAMRA, etc. at a single site in the peptide sequence. You should leave the C-terminus as a free acid (rather than amidated) if there is no acidic residue in the peptide sequence.

Obtaining Support

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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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