Molecular Devices

Product Insert

IMAP™ Purchase Plan (IPP)

Product # R8062 (Explorer Kit)
Quantity: 8000, 20 µL reactions (80 µL final volumes)

Product # R8063 (Bulk Kit)
Quantity: 50,000 20 µL reactions (80 µL final volumes)

Introduction

About the IMAP™ Explorer and Bulk Kits

The IMAP IPP Explorer and Bulk kits are available to customers participating in the IMAP Purchase Program. The kits, based on IMAP's non-antibody fluorescence polarization (FP) phosphorylation detection, can be used to evaluate enzyme activity of kinases, phosphatases, and phosphodiesterases. This assay is a simple, homogeneous "mix and read" procedure that allows accurate determination of enzyme activity.

Principle of the Assay

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 (Figure 1). This assay, unlike antibody-based kinase assays, is applicable to a wide variety of kinases without regard to the substrate peptide sequences.

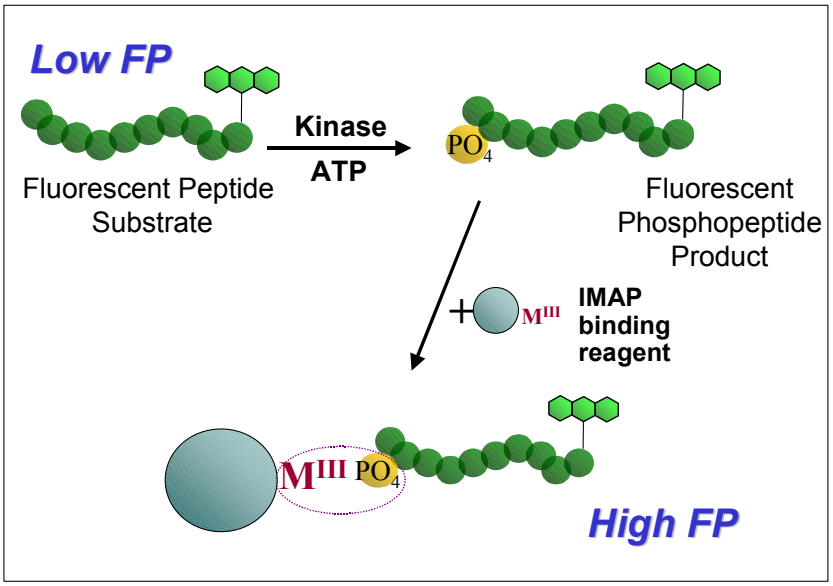

Figure 1. Principle of the IMAP system

Applications

The IMAP kits are designed for use in pharmacologic assays of kinase, phosphatase, and phosphodiesterase activity. They are especially suited for high throughput screening applications.

Materials and Equipment

Table 1: The IMAP™ Explorer Kit (P/N R8062) contains:

Reagent	Quantity	Description
IMAP™ Binding Reagent (400X)	1.5 mL	One vial of 400X binding reagent, store 4°C ****Do Not Freeze the Binding Reagent**
IMAP Binding Buffer (5x)	120 mL	One bottle, store 4°C
IMAP Reaction Buffer (5x)##	120 mL	One bottle, store 4°C. The Complete Reaction Buffer consists of a 1x stock with added DTT (1mM final reaction concentration).

Materials and Equipment

Table 2: The IMAP™ Bulk Kit (# R8063) contains:

Reagent	Quantity	Description
IMAP Binding Reagent	10 mL	One vial, store 4°C. ****Do Not Freeze the Binding Reagent**
IMAP Binding Buffer (5x)	750 mL	One bottle, store 4°C.
IMAP Reaction Buffer (5x)##	750 mL	One bottle, store 4°C. The Complete Reaction Buffer consists of a 1x stock with added DTT (1mM final reaction concentration).

##The 1x Reaction Buffer (made from the supplied 5x concentrated stock) contains 10 mM Tris-HCl, 10 mM $MgCl_2$, 0.1% BSA, 0.05% NaN_3, pH 7.2. Consult Step 1 of the Protocol for composition of Complete Reaction Buffer. Other components that can be added without affecting the IMAP system are Mn^{2+}, Ca^{2+}, DTT, 2-mercaptoethanol, certain detergents, and NaCl. The IMAP system can tolerate up to 400 μM EDTA or 40 μM EGTA without significant signal inhibition. Phosphate and structurally related molecules may compete at various affinities with the phosphopeptide for binding to the IMAP Binding Reagent; for this reason their use is not recommended. It may be that BSA binds to and interferes with the action of some inhibitory compounds. If this is the case, phosphate-free bovine gamma globulin (BGG) or detergents such as Tween-20 (0.1%) or Triton X-100 may be substituted for the BSA in the above reaction buffer formulation.

Storage and Handling

All kit components are to be stored 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.

Materials Required but not Provided

The following tables list the materials required but not supplied.

Table 3: Reagents and supplies

Reagent Item	**Source**
Black polystyrene 384-well microplate (Costar catalog #3710 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 or phosphatase enzyme	Upstate Biotechnology (recommended) or other source
Fluorescent peptide substrate	See **Appendix A**

Table 4: Compatible instruments available from Molecular Devices (MDC):

Equipment Item	**Source**
Analyst™ System with Release 2.0 Software	
One of the following: - Analyst AD - Analyst HT - Analyst GT	 MDC P/N 0200-6042 MDC P/N 0200-6044 MDC P/N 0200-6004

IMAP IPP Kit Experimental Protocol

Preparing the Reagents

This protocol should be used as an initial starting point only. Reaction concentrations, as well as other conditions, may have to be adjusted slightly for optimization of your assay system. This protocol can be done at room temperature but we recommend keeping enzyme on ice if possible prior to plate addition.

The protocol is given for 384-well microplates but can be used with 96-well or 1536-well formats (see **Appendix B**) by adjusting the volumes.

Table 5: Preparation of reagents

Step	Action
1	Prepare the **Complete Reaction Buffer**: A. Make a 1x solution of Reaction Buffer by adding 40 mL of purified water per 10 mL of the supplied 5x concentrated IMAP Reaction Buffer. When stored at 4°C, the 1x solution of Reaction Buffer is stable for six months. B. To make the Complete Reaction Buffer, add DTT to a final concentration of 1 mM in the 1x solution of Reaction Buffer. Prepare Complete Reaction Buffer fresh for each day of assay. Approximately 15 mL is required per 384 wells; scale up or down as needed.
2	Prepare the **Substrate Working Solution**: A. Prepare a 20 μM solution of the appropriate fluorescent substrate in Complete Reaction Buffer. B. Vortex gently and invert vial to make sure that all of the lyophilized substrate goes into solution. C. Add 0.1 mL of the 20 μM solution per 4.9 mL of Complete Reaction Buffer to make a 400 nM substrate working solution. This solution is 4x the final reaction concentration of 100 nM substrate.*
3	Prepare the **ATP Working Solution (kinase assays only):** A. Add 50 μL of a 10 mM stock of ATP to 1200 μL of Complete Reaction Buffer to make a 400 μM ATP stock solution. B. Add 0.25 mL of this 400 μM stock per 4.75 mL of Complete Reaction Buffer to make a 20 μM working solution. This solution is 4x the final reaction concentration of 5 μM ATP.*

4 Prepare any **enzyme inhibitors/stimulators** in Complete Reaction Buffer at 4x their final reaction concentrations. A final concentration of up to 7.5% DMSO is tolerated in the IMAP reaction.

5 Design your assay plate:

A. If you are using SOFTmax® PRO to analyze your results you may set up a template before or after reading the plate. If you set up the plate before, you can print out a template to help in pipetting your plate.

B. An example of an **enzyme dilution curve** with 3 replicates is shown below (arbitrary units):

Column→ Row ↓	1	2	3	4	5	6	7
A	0.600 units/mL	0.200 units/mL	0.067 units/mL	0.022 units/mL	0.007 units/mL	0.002 units/mL	Buffer Only control
B							
C							

C. An example of **inhibitor testing** with 3 replicates is shown below. We suggest using a concentration of enzyme that is 70% of the maximum response for your inhibitor testing:

Column→ Row ↓	1	2	3	4	5	6	7
A	enzyme + inhibitor 1	enzyme + inhibitor 2	enzyme + inhibitor 3	enzyme + inhibitor 4	enzyme + inhibitor 5	enzyme + inhibitor 6	Buffer Only control
B							
C							

6 Prepare the enzyme working stock solution:

A. To prepare an enzyme dilution curve as shown in Step 5B, make a 60 µL stock of approximately 2.400 units/mL in Complete Reaction Buffer, and serially transfer 20 µL of this stock to 40 µL of Complete Reaction Buffer. The resulting dilutions of 2.400, 0.800, 0.267, 0.089, 0.030 and 0.010 units/mL are 4x the final reaction concentrations of 0.600, 0.200, 0.067, 0.022, 0.007 and 0.002 units/mL. The actual units you use will depend on the enzyme.

7	Add components to the 384-well assay plate:* A. Add 5 µL of any inhibitors/stimulators prepared in Step 4 or add 5 µL Complete Reaction Buffer (for enzyme dilution curve as shown in Step 5B.) to the appropriate wells. B. Add 5 µL of each enzyme dilution prepared in Step 6 to the appropriate wells. For inhibitor testing use your predetermined EC_{70} enzyme dilution and incubate at this point as needed at room temperature to allow for interaction with the enzyme. C. Add 5 µL of the Substrate Solution prepared in Step 2 to the appropriate wells. D. Add 5 µL of the ATP Working Solution prepared in Step 3 to the appropriate wells. E. For the Buffer Only background control, add 20 µL of Complete Reaction Buffer to the appropriate wells. Each well of the assay should now have 20 µL volume.
8	Cover the plate and protect from light. Incubate at room temperature for 60 minutes. You may need to optimize reaction time for your individual needs.
9	Prepare sufficient **IMAP Binding Solution**: A. Make a 1x solution of Binding Buffer by adding 40 mL of purified water per 10 mL of the supplied 5x concentrated IMAP Binding Buffer. When stored at 4°C, the 1x solution of Binding Buffer is stable for six months. B. Dilute the IMAP Binding Reagent **1/400** into the 1x solution of Binding Buffer. **Store on ice until needed**. For example, if you have prepared the Enzyme Dilution Curve in Step 5B, add 4 µL of Binding Reagent per 1.6 mL of 1x Binding Buffer. You may need to optimize the concentration of Binding Reagent for your particular assay. This solution should be prepared fresh on each day of assay. C. Add 60 µL of Binding Solution to each assay well, including the Buffer Only wells.
10	Cover the plate and protect from light. Incubate at room temperature for 30 minutes. Longer incubation times can provide a slight increase in response.

11	Measure the **fluorescence polarization** (FP) on the Analyst AD, HT or GT. The suggested settings include: Continuous lamp Excitation fluorescein 485nm-20fwhm Emission 530nm-25fwhm Fluorescein 505 dichroic Z-height 3mm Attenuator out SmartRead or Comparator Sensitivity 0 Integration time of 100,000 µsec
12	Analyze your results: A. Calculate the average background (= Buffer Only wells) for both S and P fluorescent intensity data. B. Subtract the background value from both S and P raw data. C. Calculate FP and plot FP against enzyme concentration.

*** Note:** These are typical substrate and ATP concentrations that you may wish to vary according to your needs. You may combine the ATP and the substrate solutions into a single reagent added at once (Step 7).

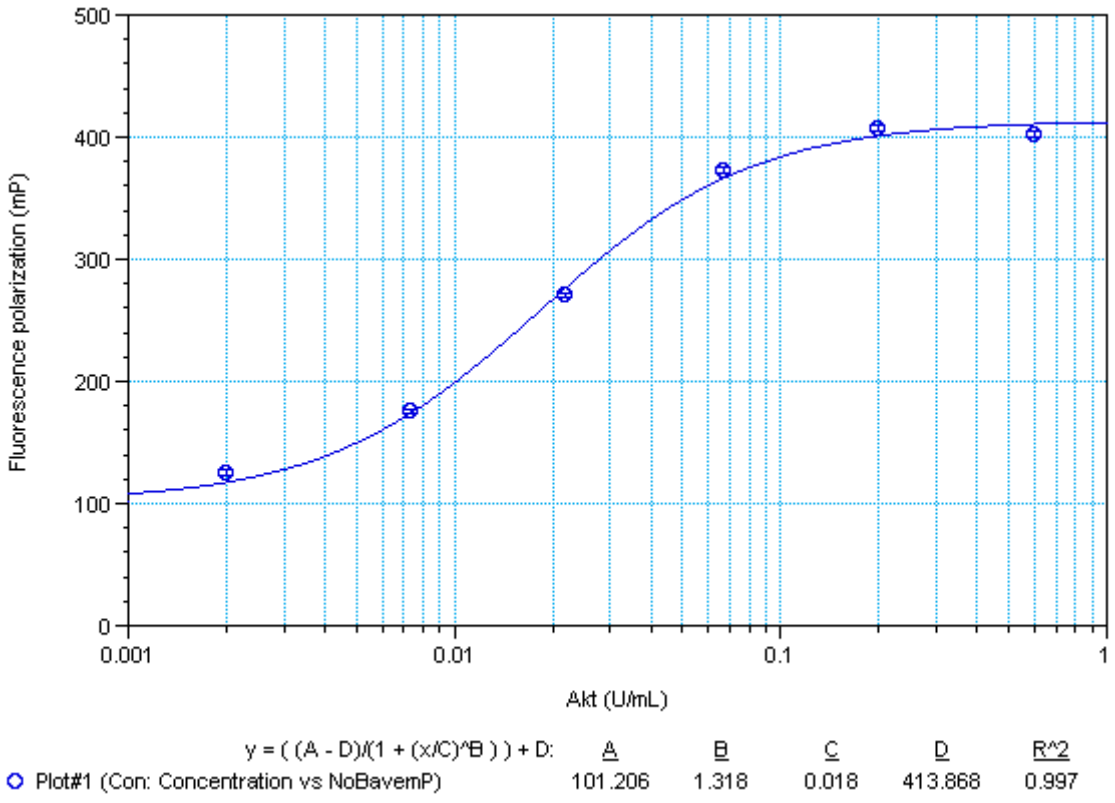

Figure 2: Representative titration of a kinase (Akt) with the IMAP™ IPP Bulk Kit (SOFTmax® PRO graph).

Appendix A. About Substrates:

The IMAP IPP Kits are designed to provide flexibility of choice in designing peptide substrates. Molecular Devices has optimized several peptide substrates that work with the IMAP system. Please refer to the IMAP Substrates Package insert and/or inquire with your Molecular Devices' technical support representatives for more information about the validated MDC and other possible substrates.

Substrate design: You can design your own fluorescent peptide substrates. In doing so, keep in mind that polarization increases with molecular weight, so that the smaller your peptide substrate is, the lower the initial polarization reading will be, hence the better your signal range will be. In general it is best to start with peptides of molecular weight less than 2500 Daltons. These should be labeled with fluorescein or other FP competent fluorophores like rhodamine 6G, TAMRA, BODIPY‡ etc. at a single site in the peptide sequence. Finally, although IMAP does not have a sequence dependence in the manner that antibody-based assays do, it is possible that certain peptides with many acidic residues (i.e., high aspartate or glutamate content) may bind non-specifically to the IMAP binding reagent. Therefore we recommend keeping the number of such residues at two or less, and using peptides with amidated C-termini when possible.

‡BODIPY is a registered trademark of Molecular Probes, Inc.

Appendix B. 1536-well format:

The Mix-and Read format of IMAP assays makes them ideal for miniaturization. Below is a sample protocol for running an IMAP kinase assay in a total volume less than 10 µL. We have simply reduced the volumes about 10-fold from the previous protocols that were done in 384-well plates.

For 1536-well homogeneous assays like this one, we recommend using the ScreenStation™, an integrated assay assembly system that combines plate stackers, low volume non-contact dispensing, and Acquest multi-mode reader.

1. Dispense compounds into the 1536-well plate. This step may be accomplished in various ways depending on your equipment and strategy for compound distribution. A few suggestions:
    a. liquid dispensing: add compounds in 1 µL volume, with DMSO content less than 20%. (This will depend on the tolerance of your enzyme for DMSO. IMAP can tolerate DMSO in excess of 10%, but most kinases are sensitive to concentrations in excess of 5% or even less.)
    b. dry-down approach: compounds may be diluted down to the target concentration (or to 0.5X of the target concentration) in ethanol, then 5 µL of this ethanol solution distributed to the assay plate. Allow the plates to dry in a clean environment (e.g., a laminar flow hood).

2. Dispense 1.0 µL kinase diluted to working concentration in reaction buffer to every well. You may want to have some wells with buffer in place of enzyme to serve as assay controls.

3. To start the reaction, add 1.0 µL substrate mixed with ATP (use concentrations suggested in 384-well protocol). Incubate for the pre-determined period of time (typically 30-60 minutes).

4. Add 6 µL of binding reagent (1:400) to stop the reaction and quantify phosphorylation. Read the plate on settings similar to those for the 384-well protocol.

For Life Science and drug discovery research use only. Not for diagnostic applications.

Molecular Devices

Molecular Devices Corporation
1311 Orleans Drive
Sunnyvale, CA 94089 USA
Email: info@moldev.com
www.moleculardevices.com

Sales Offices

USA 800-635-5577 • **UK**+44-118-944-8000 • **Germany** +49-89-9620-2340 • **Japan** +06-6399-8211
Check our web site for a current listing of our worldwide distributors.

R3307 Rev. B