IMAP Screening Express Assay Kit

The IMAP® Screening Express Kit is used to evaluate enzyme activity of a wide variety of protein kinases, phosphatases, and phosphodiesterases based on IMAP's non-antibody fluorescence polarization (FP) detection of phosphorylation. This assay is a simple, homogeneous mix and read procedure that allows accurate determination of enzyme activity.

Available Kit

	Data Points	Kit
IMAP FP Screening Express Kit	8,000, 20 µL reactions (80 µL final volumes)	R8073

Contents

R3340 C
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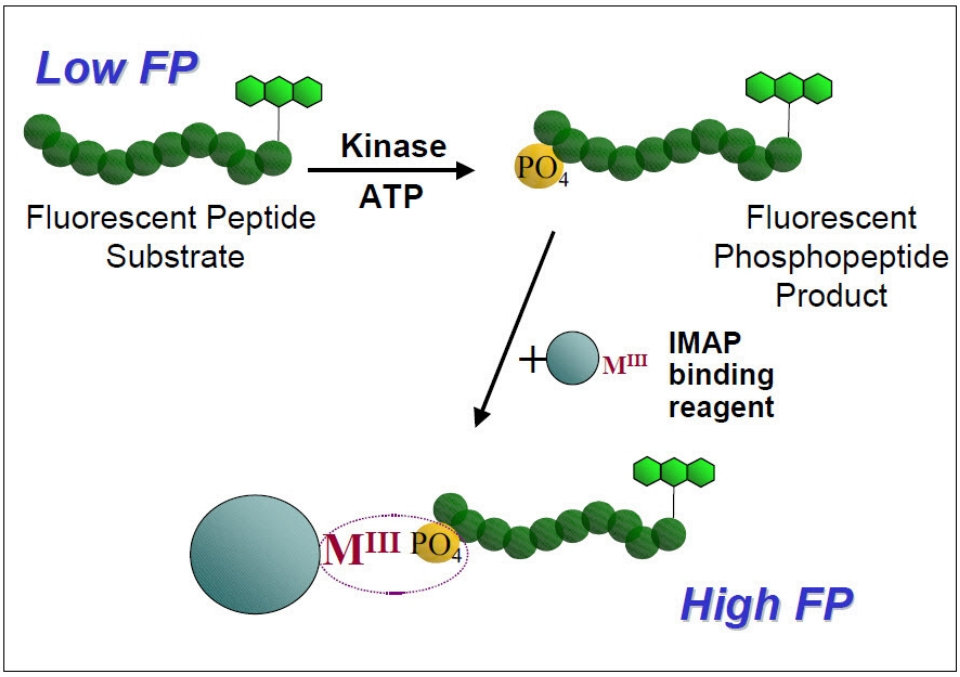
MOLECULAR DEVICES

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

Product manufactured by Molecular Devices, LLC.
3860 N. First Street, San Jose, California, 95134, United States of America.
Molecular Devices, LLC is ISO 9001 registered.

Chapter 1: 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 System

Applications

The IMAP Screening Express Assay Kit is designed for use in pharmacologic assays of kinase, phosphatase, and phosphodiesterase activity. It is especially suited for high throughput screening applications.

Chapter 2: Materials and Equipment

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Kit Components

Components of the IMAP Screening Express Assay Kit

Reagent	Quantity	Description
IMAP Binding Reagent	1.5 mL	One vial, 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 (1 mM 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.
For composition of Complete Reaction Buffer, see Optimization Protocol on page 7.

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 or phosphatase enzyme	Upstate Biotechnology or other source
Fluorescent peptide substrate	See About Substrates on page 11

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 Screening Express Assay Kit 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® M5 Multi-Mode Microplate Reader
- SpectraMax® M5e Multi-Mode Microplate Reader
- SpectraMax® Paradigm Multi-Mode Microplate Reader

Chapter 3: Optimization Protocol

Use this protocol as an initial guide only. Adjust the reaction 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. 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. Therefore, their use is not recommended. If the BSA binds to and interferes with the action of some inhibitory compounds, you can substitute phosphate-free bovine gamma globulin (BGG) or detergents such as Tween-20 (0.01%) or Triton X-100 for the BSA in the reaction buffer formulation.

The protocol is for 384-well plates. Adjust the volumes for 96-well or 1536-well plates. See 1536-Well Plates on page 13.

To run the experimental protocol:

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 fluorescent substrate in Complete Reaction Buffer.
   b. Vortex gently and invert vial to make sure that all 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 the assay plate:
   a. Use the SoftMax® Pro Data Acquisition and Analysis Software to analyze the results. You can 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 you pipette the plate.
   b. The following is an example of an enzyme dilution curve with 3 replicates (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. The following is an example of inhibitor testing with 3 replicates. You should use a concentration of enzyme that is 70% of the maximum response for the inhibitor testing.

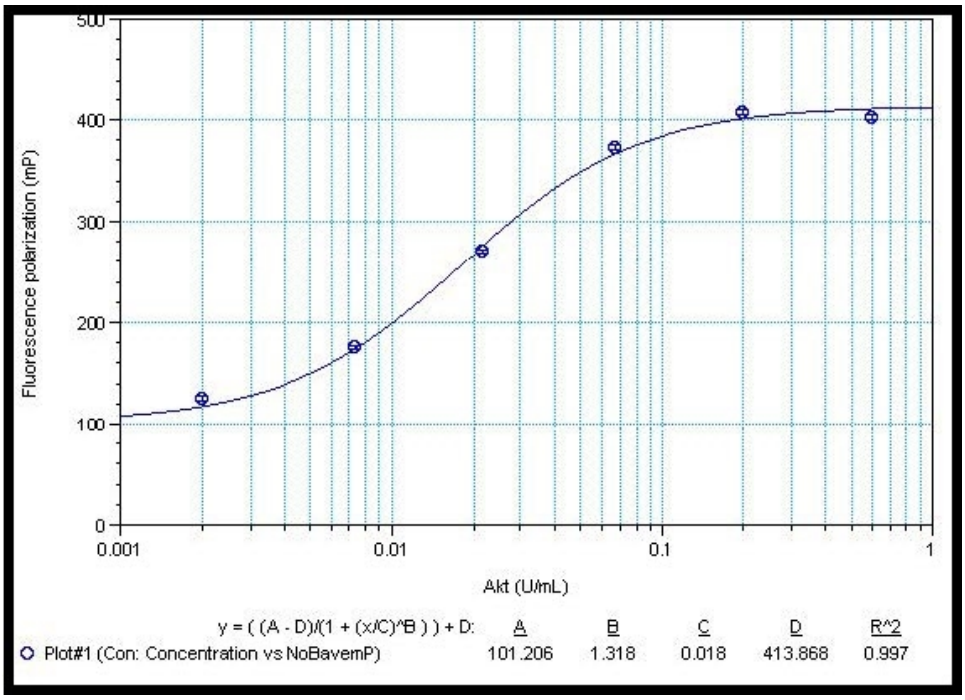
Column→ Row ↓	1	2	3	4	5	6	7
A	enzyme + inhibitor 1	enzyme + inhibitor 2	enzyme + inhibitor 3	enzyme + inhibitor 4	enzyme + inhibitor 5	enzyme + inhibitor 6	Buffer Only control
B							
C							

6. Prepare the enzyme working stock solution: 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 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 the 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 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 prepare 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 assay. You should prepare this solution 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. The SoftMax Pro Software settings to read the plate depend upon the microplate reader you use. See the *Instrument Settings* document for details.
12. Analyze the 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 can vary according to your needs. You can combine the ATP and the substrate solutions into a single reagent added at once (step 7).

Chapter 4: Sample Data



Representative Titration of a Kinase (Akt) with the IMAP Screening Express Kit

About Substrates

The IMAP Screening Express Kit is designed to provide flexibility in designing peptide substrates. Molecular Devices has optimized several peptide substrates that work with the IMAP system. See the *IMAP Substrates* document.

You can design your own fluorescent peptide substrates. Polarization increases with molecular weight, so that the smaller the peptide substrate, the lower the initial polarization reading, hence the better the signal range. In general, you should start with peptides of molecular weight less than 2500 Daltons. You should label these with fluorescein or other FP competent fluorophores like rhodamine 6G, TAMRA etc. at a single site in the peptide sequence. Although IMAP does not have a sequence dependence in the manner that antibody-based assays do, certain peptides with many acidic residues (for example, high aspartate or glutamate content) may bind non-specifically to the IMAP binding reagent. You should keep the number of such residues at two or less and use peptides with amidated C-termini when possible.

Appendix A: 1536-Well Plates

The mix and read format of IMAP assays makes them ideal for miniaturization. The following is a sample protocol to run an IMAP kinase assay in a total volume less than 10 µL. This reduces the volumes about 10-fold from the previous protocols that were done in 384-well plates.

To run the protocol using a 1536-well microplate:

1. Dispense compounds into the 1536-well plate. You can accomplish this in various ways depending on the 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 depends on the tolerance of the 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: dilute compounds down to the target concentration (or to 0.5X of the target concentration) in ethanol, then distribute 5 µL of the ethanol solution to the assay plate. Allow the plates to dry in a clean environment (for example, 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.

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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