Multiplexing and Cascade Assays with the IMAP Fluorescence Polarization Platform

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Abstract

The IMAP® fluorescence polarization (FP) assay platform is a generic, homogeneous system applicable to a variety of enzymes, including protein kinases. IMAP is based on the high affinity binding of phosphate to immobilized trivalent metals. Dozens of kinases from throughout the kinome have been successfully assayed with IMAP, and a wide variety of kinase-specific TAMRA- and FAM-labeled substrates are now available from Molecular Devices. Here we show how to develop multiplexed assays with IMAP, utilizing both red- and green-labeled peptides in the same well. We also discuss "cascade", or "coupled", IMAP assays, in which an active kinase activates an inactive kinase or kinases, leading to downstream phosphorylation of a fluorescent peptide and high polarization values. These data will also highlight Molecular Devices' new IMAP Progressive Binding System, which enables the HTS researcher to optimize their assay for each application.

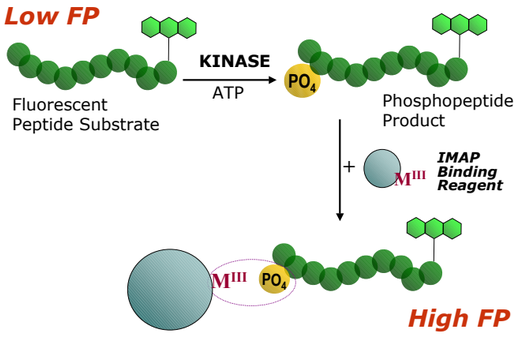

Figure 1. The IMAP technology is based on the high affinity binding of phosphate by immobilized metal (M^{III}) coordination complexes on nanoparticles. This IMAP "binding reagent" complexes with phosphate groups on phosphopeptides generated in a kinase reaction. Such binding causes a change in the rate of the molecular motion of the peptide, and results in an increase in the FP 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. IMAP can be also used to analyze phosphatase activity, simply by starting out with fluorescent phosphopeptide as the substrate.

The IMAP Progressive Binding System

The Progressive Binding System is configured to enable the researcher to achieve maximum performance for **every possible application**, by altering the ratios of Progressive Buffers A and B and the concentration of Progressive Binding Reagent.

Reagent	Description
IMAP Progressive Binding Buffer A	Baseline binding buffer
IMAP Progressive Binding Buffer B	Affects FP 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 covalent coordination complex bond.

Table 1. The three components of the IMAP Progressive Binding System. By balancing the proportions of Buffer B and the Binding Reagent, you can optimize the binding solution to provide ample binding sites without increasing background FP, for even very acidic peptides. All peptide substrates offered by Molecular Devices are pre-optimized and their recommended assay conditions are provided in the package insert.

Multiplexing with IMAP: PAR1Bα and Nek2

The IMAP assay platform is highly amenable to multiplexing with multiple enzymes using different wavelength dye-labeled substrates. IMAP substrates have been optimized for each enzyme we test, and in many cases are highly specific for a particular enzyme. Here the Ser/Thr kinases PAR1Bα and Nek2 are assayed simultaneously in the same wells, then read with different methods on the Analyst instrument. The Progressive Binding System allows researchers to "tune" the ΔmP signal to maximize multiplex performance, as summarized in the multiplex curves in Graphs A and B. The assay with the optimal binding solution is shown in Graphs C and D. **Multiplexed results are identical to results determined individually**.

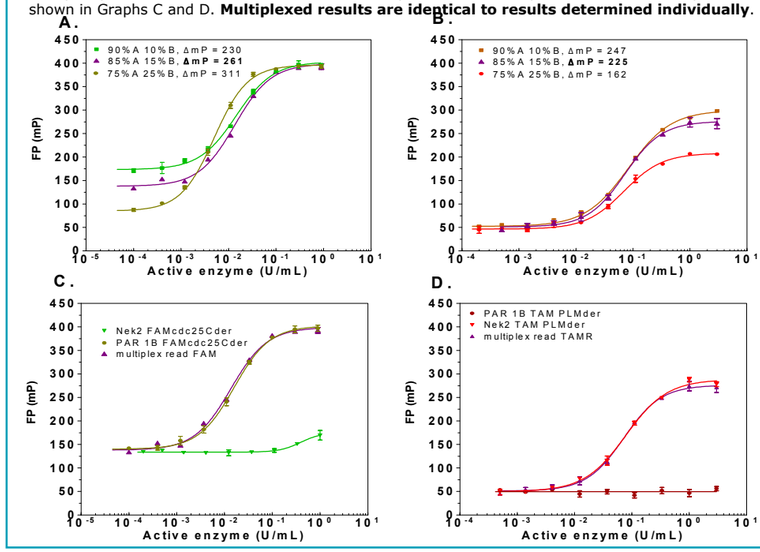

Figure 2. PAR1Bα and Nek2 IMAP multiplex assay. The three different Progressive Binding Buffer proportions tested were 90%A and 10%B, 85%A and 15%B, and 75%A and 25%B, all with 1/400 Progressive Binding Reagent. Curves were read with the Analyst 485/505/530 filter set (A, C), or the 530/561/590 filter set (B, D). To compare the different binding solutions, only the multiplex curves containing both enzymes and both substrates are shown in graphs A and B. The full set of curves assayed with the 85%A 15%B 1/400 Binding Reagent is shown in C and D. All titrations contained 100 µM ATP in the 1 hour reaction. C: enzyme titration with 100 nM cdc25C-derived substrate (5FAM-VSRSGLYRSPSMPENLNRPR-COOH), read with the 485/505/530 filter set. D: enzyme titration with 100 nM PLM-derived substrate (5TAMRA-IRRLSTRRR-COOH), read with the 530/561/590 filter set. In graphs C and D, purple triangles indicate the multiplex assay. Error bars indicate mP standard deviations of 2 replicates. The FP standard deviations (SDs) ranged from 1-13 mP, with the average SD = 4. The Z' factors for all multiplex curves were >0.67 at 0.14 U/mL Nek2, and >0.73 at 0.016 U/mL PAR1Bα.

Multiplexing with IMAP: MAPKAP K1b and MAPKAP K2

In this experiment, the Ser/Thr kinases MAPKAP1b (Rsk2) and MAPKAP K2 are assayed simultaneously in the same wells, then read with different methods on the Analyst instrument. Although both enzymes can phosphorylate both substrates, the concentrations of each can be adjusted to avoid cross-reactivity.

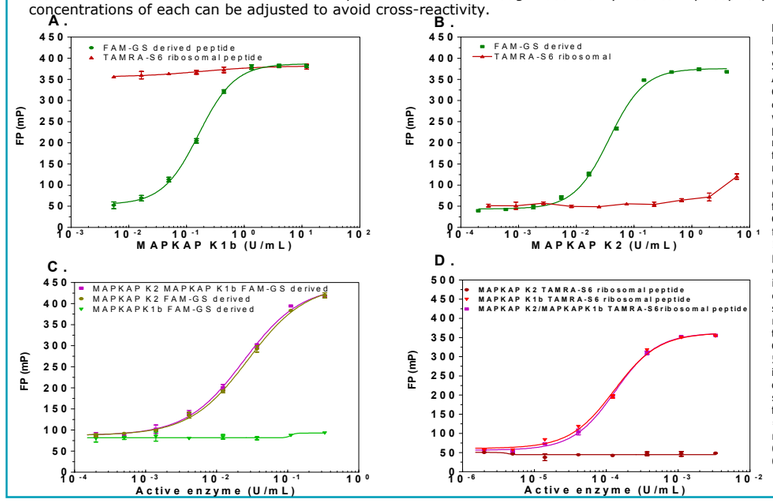

Figure 3. MAPKAP 1b (Rsk2) and MAPKAP K2 IMAP multiplex assay with IMAP Progressive Binding System (100% Progressive Buffer A, 1/400 Binding Reagent). A,B: Check to determine non-crossreactive enzyme concentration with 100 nM FAM-GS-derived peptide(5FAM-KKLNRTLSVA-COOH) read with the Analyst 485/505/530 filter set and 100 nM TAMRA-S6 ribosomal-derived-peptide (5TAMRA-AKRRRLSSLRA-COOH) read with the Analyst 530/561/590 filter set. Graph A, MAPKAP K1b. Graph B, MAPKAP K2. C,D: The five enzyme titrations contained 100 µM ATP and 100 nM of each peptide in the 1 hour reaction. In each graph, the purple squares indicate the multiplex assay, which contained both enzymes and both substrates (200 nM total peptide in multiplex wells). Graph C read with the 485/505/530 filter set and Graph D read with the 530/561/590 filter set. Error bars indicate the mP standard deviations of 4 replicates. The FP standard deviations (SDs) ranged from 1-11 mP, with the average SD = 4. The Z' factors for the multiplex assays were >0.83 at 0.0004 U/mL MAPKAP K1b and at 0.012 U/mL MAPKAP K2.

IMAP Cascade Assays: B-Raf® MEK1® Erk2® 5FAM-Erktide

For some enzymes, an efficient peptide substrate is not readily available. In these cases, one can measure kinase activity with a downstream kinase which does phosphorylate a peptide, and **couple the target enzyme's activity to that downstream kinase**. Here we use active B-Raf to activate MEK1, which in turn activates Erk2, which then phosphorylates the substrate in an IMAP assay.

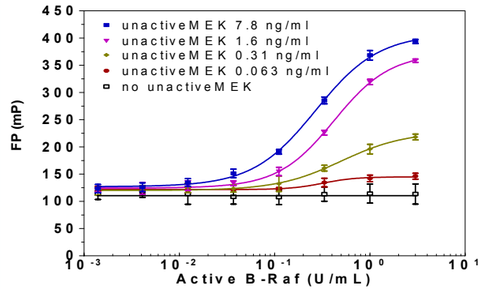

Figure 4. B-Raf cascade assay with IMAP Progressive Binding System. Active B-Raf was titrated with different concentrations of unactive MEK1, as indicated, and unactive Erk2 (700 ng/mL) with 100 nM FAM-Erktide (IPTTPITTTYFFFK-5FAM-COOH) and 100 µM ATP in a 1 h reaction. The IMAP reaction buffer contained 0.01%Tween20 substituted for 0.1%BSA. The binding solution used was 75%A, 25%B, 1/600 Progressive Binding Reagent. Curves were read with the Analyst 485/505/530 filter set The error bars indicate the mP standard deviations of 4 replicates. The FP SDs ranged from 1-9 mP, and for the assay with 7.8 ng/mL unactive MEK1 the Z' factor was >0.7 at 0.1 U/mL.

IMAP Cascade Assays: PDK1 SGK1 5FAM-Crosstide

The Ser/Thr kinase PDK1 activates SGK1, which in turn phosphorylates the Crosstide substrate.

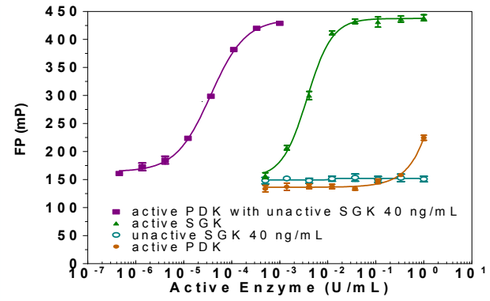

Figure 5. PDK1 cascade assay with IMAP Progressive Binding System. Active PDK1 was titrated with 40 ng/mL unactive SGK1, as indicated, and control titrations of unactive SGK1 (40 ng/mL) alone, active PDK1 alone, or active SGK1 alone are also shown. All reactions were 1 h and contained 100 µM ATP and 100 nM 5FAM-Crosstide substrate (5FAM-GRPRTSSFAEG-COOH). The binding solution used was 75%A, 25%B, 1/600 Progressive Binding Reagent. Curves were read with the Analyst 485/505/530 filter set The error bars indicate the mP standard deviations of 4 replicates. The FP SDs ranged from 1-9 mP, and the Z' factor for the cascade assay (purple squares) was >0.8 at 3.7 x 10^{-5} U/mL.

Summary

Multiplexing with IMAP:

- 2+ kinases assayed simultaneously
- TAMRA and Fluorescein make perfect multiplexing partners
- Other labels with distinct fluorescent spectra can be used
- Progressive Binding System allows for high concentrations of ATP (up to 1 mM)

Cascade Assays with IMAP:

- For kinases best assayed with the physiological substrate
- Can increase assay sensitivity

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