Multiplex assays in IMAP using the Progressive Binding System

#A7

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. It has been applied to a wide variety of kinases spanning the whole kinome. Assays using FAM- labeled peptides as well as TAMRA- or other red-labeled peptides have been developed. The recently introduced Progressive Binding System enables the researcher to optimize the IMAP binding solution specifically for each assay substrate. Here we show how to develop multiplexed assays with IMAP, utilizing both red- and green-labeled peptides in the same well. In this way, two different kinases can be assessed in the same assay. We also discuss how peptides with very different binding solution requirements can work together in a multiplexed assay.

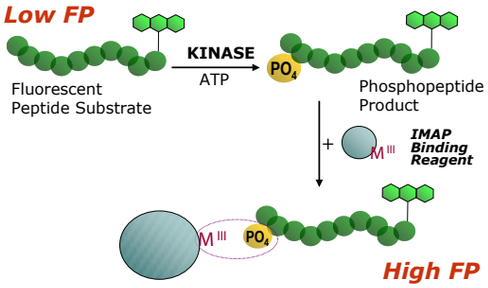

Figure 1. The IMAP technology is based on the high affinity binding of phosphate by immobilized metal (M^{III}) coordination complexes on nanoparticles. This IMAP "binding reagent" complexes with phosphate groups on phosphopeptides generated in a kinase reaction. Such binding causes a change in the rate 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.

Development and Set-Up of an IMAP Multiplexed Assay

Row	Serial Dilutions of Enzyme	Substrate	Multiplex [ATP]	Expected Result
1	Kinase 1	FAM-peptide 1	[optimal ATP] for Kinase 1 + [optimal ATP] for Kinase 2 for each curve	1: High FP signal
2	Kinase 2	FAM-peptide 1		2: No FP signal
3	Kinase 1	TAMRA-peptide 2		3: No FP signal
4	Kinase 2	TAMRA-peptide 2		4: High FP signal
5	Kinase 1+ 2	FAM-pep 1 + TAMRA-pep 2		5: High FP both filter sets

Table 1. Assay template of target enzymes and substrates to optimize multiplexing conditions. All different enzyme/combinations are assayed to validate the multiplexing results. The "multiplex ATP concentration" is defined as the sum of the optimal [ATP] for each target enzyme. The plate is read twice using the appropriate filter sets for each substrate. It is recommended that no ATP or no enzyme control assay wells are included in the assay to determine the substrate only FP. **See Example curves in Figure 4.**

Test Wells containing FAM-peptide 1+ TAMRA-peptide 2 + multiplex [ATP]		Binding Solution
Kinase 1 + Kinase 2	No enzyme	Binding Solution with largest proportion of Buffer B
Kinase 1 + Kinase 2	No enzyme	Alternative Binding Solution 1
Kinase 1 + Kinase 2	No enzyme	Alternative Binding Solution 2

Table 2. Template to assess ΔmP signal with different Progressive Binding Solutions. Each substrate is tested +/- EC_{70} [enzyme] in the presence of the multiplex [ATP]. FP is read twice, with appropriate filters. The Progressive Binding System can be used for any peptide substrate.

IMAP Assays with Protein Substrates: Histone H1

For some enzymes, the most efficient substrate is a small protein, not a peptide. Because FP assays rely on the difference in molecular rotation of molecules that differ in molecular volume, which loosely correlates with molecular weight, fluorescently labeled substrates must be relatively small to produce a measurable FP signal. **Some fluorescently labeled proteins have been successfully used in IMAP. Histone H1 is a widely used Ser/Thr kinase substrate, and provides a 10x more sensitive assay than the peptide substrate.**

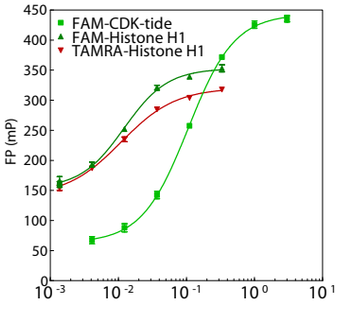

Figure 2. 100 nM FAM-CDKtide (5FAM-GGGPAPKKAKKL-$CONH_2$), and 150 nM FAM- or TAMRA-labeled Histone H1 were compared. Each 1h reaction contained 100 µM ATP. For FAM-CDKtide and TAMRA-Histone H1 assays, binding solution was 100% Buffer A, 1/400 Progressive BR; for FAM-Histone H1, 85% A, 15% B, 1/400 Progressive BR was used. After 1h, FP was read (FAM: 485/505/530, TAMRA: 530/561/590).

Protein Labeling Method:
200 µL Histone H1 (purified calf thymus, 32 kDa by SDS-PAGE, Upstate) was mixed in 1:1 molar ratio with 5TAMRA or 5FAM (succinimide ester) in carbonate buffer pH 9.6. After 1.5 h incubation RT in the dark, 20 µL 100 mM Tris pH 7.2 was added and incubated a further 2h. Each labeled protein was purified on a PD-10 desalting column (Amersham) equilibrated with 10 mM Tris pH 7.2. 30 fractions (0.5 –1mL) were collected and the highest intensity fraction was used in the assay.

IMAP Progressive Multiplexing: CHK1 and CDK5/p25

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 CHK1 and CDK5/p25 are assayed simultaneously in the same wells, then read with different methods on the Analyst instrument. **Multiplexed results are identical to results determined individually**.

Numbers correspond to Table 1 Expected Results

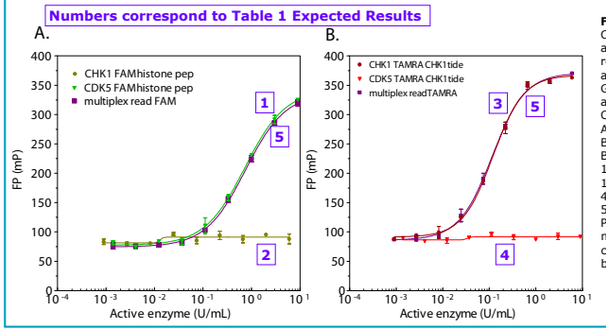

Figure 4. CHK1 and CDK5/p25 titrations set up as in Table 1 in a 1 h reaction with 250 µM ATP and 100 nM CDKtide (5FAM-GGGPAPKKAKKL-$CONH_2$) and/or 100 nM TAMRA-CHK1tide (TAMRA-ALKLVRYPSFVITAK-COOH). Binding Solution was 75% Buffer A, 25% Buffer B, and 1/400 Progressive BR. After 1h, FP was read: 485/505/530 FAM (A), 530/561/590 TAMRA (B). Purple squares indicate the multiplex assay, which contained both enzymes and both substrates.

IMAP Progressive Multiplexing: PAR1Bα and Nek2

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.

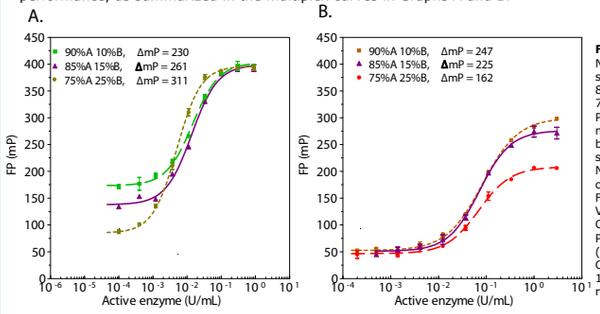

Figure 5. PAR1Bα and Nek2 multiplex with binding solutions 90%A/10%B, 85%A/15%B, and 75%A/25%B, (all 1/400 Progressive BR). Only multiplex curves containing both enzymes and both substrates are shown. Neither substrate was cross-reactive. 100 nM FAM-cdc25C-derived (FAM-VSRSGLYRSPSMPENLNRPR-COOH), 100 nM TAMRA PLM-derived substrate (5TAMRA-IRRLSTRRR-COOH) were assayed with. 100 µM ATP in a 1 hr reaction.

IMAP Original Multiplexing: MAPKAP K2 and IKKβ

The Original Binding System allows researchers to simply add a set concentration of Original Binding Reagent to Binding Buffer, incubate for 30 minutes, and read the FP. This system is for enzymes requiring < 30 µM ATP and peptide substrates containing <3 acidic residues. For other conditions, the Progressive system is recommended.

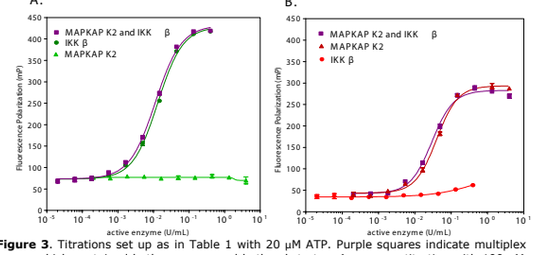

Figure 3. Titrations set up as in Table 1 with 20 µM ATP. Purple squares indicate multiplex assay, which contained both enzymes and both substrates. A: enzyme titration with 100 nM IκBα-derived peptide (5FAM-GRHDSGLDSMK-$CONH_2$), read on 485/505/530. B: enzyme titration with 20 nM GS-derived peptide (5TAMRA-KKLNRTLSVA-COOH), read on 530/561/590.

IMAP Progressive Multiplexing: MAPKAP K1b and MAPKAP K2

Although both MAPKAP1b (Rsk2) and MAPKAP K2 enzymes are closely related and can phosphorylate both substrates, the concentrations of each can be adjusted to avoid cross-reactivity.

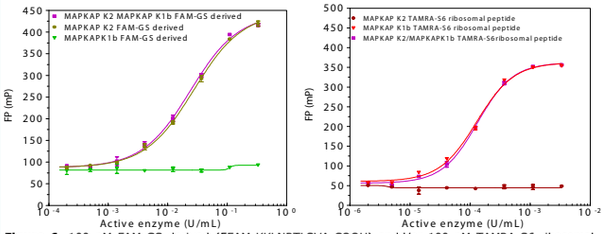

Figure 6. 100 nM FAM-GS-derived (5FAM-KKLNRTLSVA-COOH) andd/or 100 nM TAMRA-S6 ribosomal-derived (5TAMRA-AKRRRLSSLRA-COOH) assayed with 100 µM ATP in 1h reaction. Purple squares indicate multiplex assay.

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Using the IMAP Substrate Finder to Determine Substrates for Multiplexing: PKC isoforms

The IMAP Substrate Finder plate enables the researcher to quickly identify suitable substrates for their target kinase. 59 fluorescein-labeled peptides are arrayed in quadruplicate in a ready-to-use concentration on the plate. The researcher adds ATP and +/- enzyme, incubates, adds binding solution, and then reads the FP. The peptide substrates in this first plate target the CamK/AGC family of Kinases.

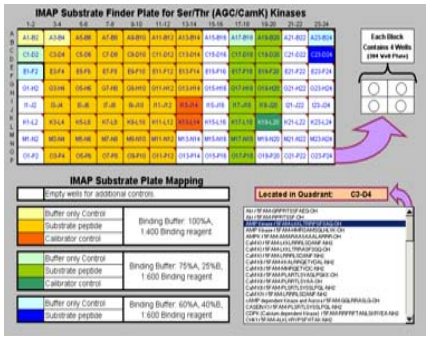

Figure 7 (above). The IMAP Substrate Mapper interface. The Mapper is included with the Substrate Finder kit and displays the plate layout, the peptide sequences with their known targets, and the original PubMed reference for each.

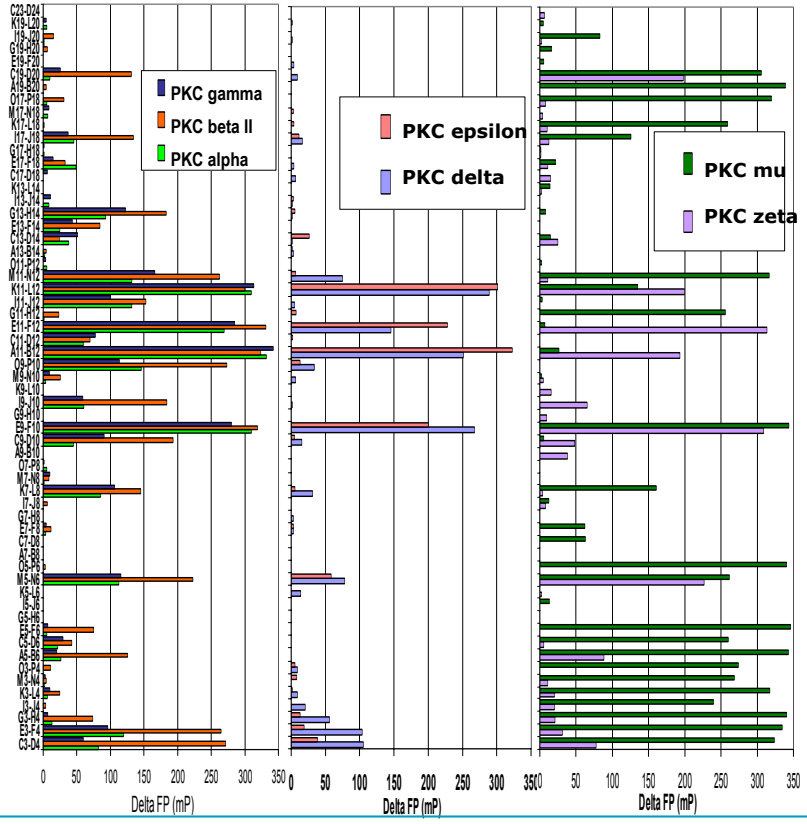

Figure 8 (right). Signal profiles generated with the Substrate Finder for the indicated PKC isoforms (Upstate). The delta mP achieved with each peptide (listed on the long axis by plate well ID) is shown. For PKCβII, 1 U/mL of enzyme was used; for the other isoforms, 0.75 U/mL was used. The 1h reactions contained 100 nM substrate and 100 μM ATP. PKCα, PKCβII, and PKCγ assays contained 100 μM $CaCl_2$. Three different Binding Solutions were used, according to the kit directions. Phospholipid and DAG were not included in the assay, but improve assay sensitivity when added (data not shown).

Progressive Multiplexing: PKC zeta and PKC mu

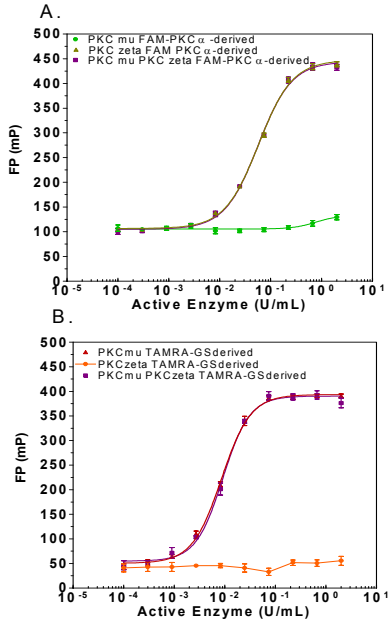

Figure 5. Enzyme titrations set up as in Table 1 in a 1 h reaction with 100 μM ATP and 100 nM FAM PKCα-derived (5FAM-RFARKGSLRQKNV-COOH) and/or 100 nM TAMRA-GS-derived (TAMRA-KKLNRTLSVA-COOH) peptides. Binding Solution was 100% Buffer A, 1/400 Progressive BR. After 30min, FP was read: 485/505/530 FAM (A), 530/561/590 TAMRA (B). Purple squares indicate the multiplex assay, which contained both enzymes and both substrates.

Progressive Multiplexing with the inhibitor H89: CHK1 and Akt1

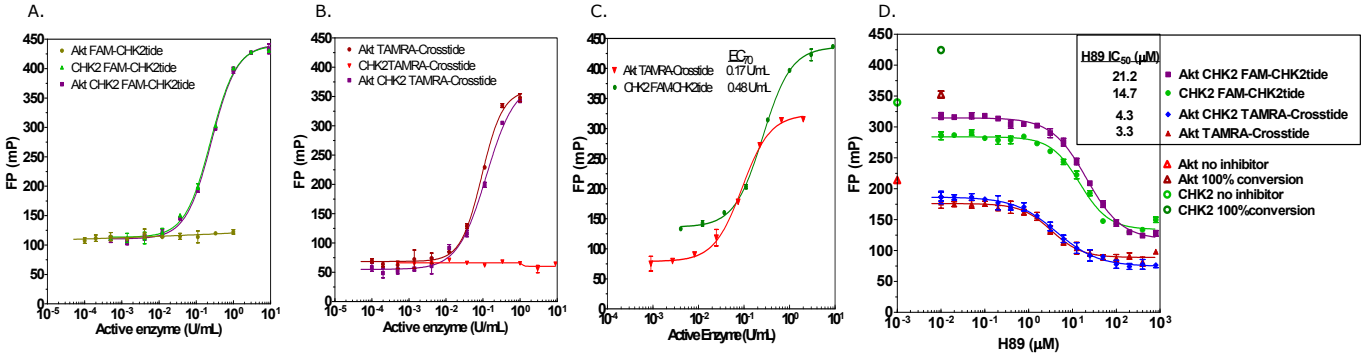

Figure 5. A, B. Enzyme titrations set up as in Table 1 in a 1 h reaction with 100 μM ATP and 100 nM FAM CHK2tide (5FAM-AMRLERQDSIFYPK-$CONH_2$) and/or 100 nM TAMRA-Crosstide (TAMRA-GRPRTSSFAEG-COOH) peptides. Binding Solution was 75% Buffer A, 25% Buffer B, 1/400 Progressive BR. After 1h, FP was read: 485/505/530, FAM (A), 530/561/590, TAMRA (B). Purple squares indicate the multiplex assay, which contained both enzymes and both substrates. C. EC_{70} determination in reaction buffer containing 0.01% Tween20 instead of 0.1% BSA, binding solution 70%A/30%B, 1/600 Progressive BR. D. EC_{70} concentrations of Akt (0.16 U/mL) and CHK2 (0.48 U/mL) were tested in the presence of two-fold dilutions of H89 inhibitor (Calbiochem) in a 30 minute reaction with 100 μM ATP in Tween reaction buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 0.01% Tween-20, 0.05% NaN_3, pH 7.2) containing 1 mM DTT. In separate wells, CHK2 was tested with 100 nM CHK2tide, green circles, and Akt was tested with 100 nM TAMRA-Crosstide, red triangles. Progressive Binding Solution was 70% A/30% B, and 1/600 Progressive BR. The multiplex assay read with the 485/505/530 filter set (purple squares) or the 530/561/590 filter set (blue diamonds) contained both enzymes and both substrates. FP of maximal enzyme (1 U/mL Akt, 9 U/mL CHK2) labeled "100% conversion". FP of EC_{70} enzyme DMSO carrier only labeled "no inhibitor". The FP of Akt no inhibitor sample was equivalent to 55% conversion of substrate to product; the FP of CHK2 no inhibitor was 71% conversion. The multiplex assay conversion rates were 55% (Akt) and 79% (CHK2).

Summary

Multiplexing with IMAP:

- **Two or more kinases assayed simultaneously.**
- **TAMRA and Fluorescein make perfect multiplexing partners.**
- **Other labels with distinct fluorescent spectra can be used.**
- **Substrate Finder is useful for identifying optimal substrates for multiplexing.**
- **Even small proteins such as Histone H1 can be used as substrates in the IMAP system.**
- **Progressive Binding System allows for high concentrations of ATP (up to 1 mM) and substrate flexibility.**

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