

Expansion of IMAP™ to assays requiring high-ATP and use of substrates with a high proportion of acidic residues.

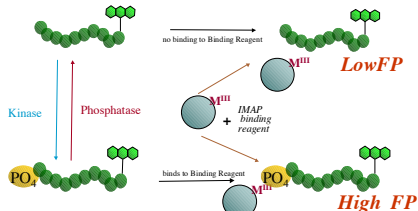
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Abstract

IMAP is a homogenous, generic, FP based HTS system used to measure kinase, phosphatase and phosphodiesterase enzyme activity without the need for antibodies. The IMAP technology is based on the high affinity binding of phosphate by immobilized metal (M^{III}) coordination complexes on nanoparticles. In this study we show approaches to increasing the ATP tolerance of IMAP and to the use of peptide substrates with a high content of acidic amino acids. With the Progressive Binding System, a recent addition to the IMAP family, one can extend IMAP to include kinases that have been difficult to assay by this approach previously. Issues of type and placement of acidic amino acids in the substrate sequence are also discussed.

Principle of the IMAP assay

The IMAP technology is based on the high affinity binding of phosphate by immobilized metal (M^{III}) coordination complexes. This IMAP "binding reagent" complexes with phosphate groups on phosphopeptides. 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. A kinase assay starts with non-phosphorylated peptide substrate and the increase of the phosphorylated species is monitored by the increase of FP signal. In a phosphatase assay the reaction starts with a phosphorylated substrate and its dephosphorylation is monitored. This assay, unlike antibody-based kinase assays, is applicable to a wide variety of phosphate transferring enzymes without regard to the substrate peptide sequences.



Components of the Progressive Binding System and their functions

Reagent	Role
Progressive Binding Buffer A	Baseline Binding Buffer
Progressive Binding Buffer B	Reduces FP background by blocking undesired interactions
Progressive Binding Reagent	Introduces the Binding entities (Metal/beads). This Binding Reagent specifically binds to phosphate residues via a covalent coordination complex bond.

Optimizing with the Progressive Binding System

The optimum proportions of the progressive binding system to use depend primarily on the acidic character of the peptide substrate. Table 1 gives guidelines for the recipes as a function of number of carboxyl groups in the substrate

No. COOH	Binding Solution to use (approx.)	Binding Incubation Time
0, 1	100% Buffer A. Binding reagent 1: 800	30 minutes
2, 3	75% Buffer A, 25% Buffer B. Binding reagent 1: 800	30 minutes
4	50% Buffer A, 50% Buffer B. Binding reagent 1: 1500	2 hours
5+	25% Buffer A, 75% Buffer B. Binding reagent 1: 2500	>6h

The guidelines of Table 1 reflect a balance between opposing effects: Increasing the amount of Buffer B generally reduces background binding, but may also reduce maximum binding, while increasing the binding reagent concentration generally increases these parameters. In addition, time for equilibration of the final assay and tolerance for ATP concentration are affected.

Kinase assays with High [ATP]

Figure 2a shows a comparison of the original IMAP system with the progressive one in an assay for CDK2. Five-fold higher ATP can be used because of the higher capacity of the new system, giving more sensitivity and dynamic range.

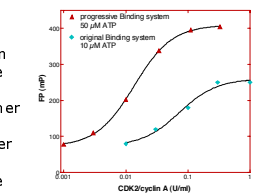


Figure 2a: **CDK2/cyclinA** assay in IMAP. Assay conditions: 100 nM substrate (5FAM-GGGPATPKKAKKL-COOH) and ATP as indicated, 1h RT. Binding reagent dilution was 1:200 for the original Binding system, 1:400 for the progressive Binding system (100% A)

Figure 2b illustrates the tolerance of the system for ATP. In this assay for CDK2, ATP concentrations up to 300 μM did not significantly interfere with the binding step.

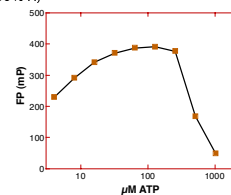


Figure 2b. **CDK2/cyclinA** ATP dependency. CDK2/cyclin A (0.06 U/ml), with binding system of 100% A and 1:400 binding reagent

IMAP Improved Binding solution stability

The new IMAP Progressive Binding system has excellent stability when constituted into working solutions. Figure 5 shows that the binding solution gives equivalent performance whether freshly made or after standing at room temperature overnight. In contrast, the original binding solution shows some instability with one of the peptides tested, the PKAtide substrate.

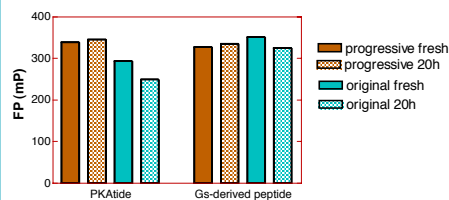


Figure 3: Comparison of 20h old Binding solution and fresh Binding solution using the original and the progressive Binding system. Shown is the response to the phosphorylated forms of the PKAtide and MAPKAP peptide substrate (GS-derived peptide).

p38 IMAP assay with the progressive Binding system

The EGF-R derived peptide **LVEPLTPSGEAPNQK(5FAM)COOH** is an example of a substrate with 3 acidic residues.

Figure 4 shows a p38β2 dilution curve read 1h and 18h after Binding solution addition, stating that also with the progressive Binding system the stability of the signal is excellent. The Figure 4 insert shows the ATP dependency of the p38 assay. With this Binding solution the assay can be run at 100 μM ATP.

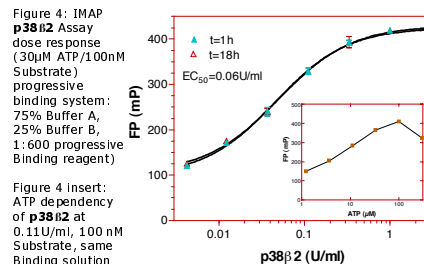


Figure 4 insert: ATP dependency of **p38β2** at 0.11U/ml, 100 nM substrate, same Binding solution

Highly acidic substrates

The preferred peptide substrate for Casein Kinase 2 (CK2) has the sequence RRRADSDDDDD and is one of the most commonly used acidic kinase substrates. Casein Kinase 2 is emerging as a preferred drug target, so it was selected for evaluation with the IMAP Progressive Binding system. Figure 3 shows that the background was very high and could not be controlled by adjusting Progressive Buffer B alone. With additional NaCl, however, a buffer system is created that gives acceptable background.

In Figure 4, these conditions are applied to the assay of CK2. This assay response was virtually unattainable with the original IMAP binding system.

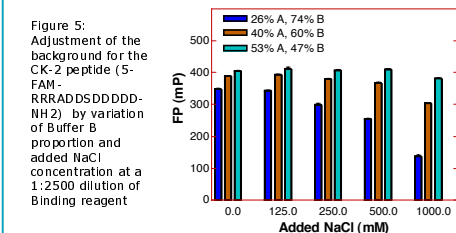
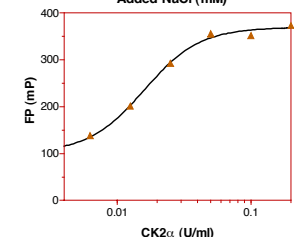


Figure 6: IMAP CK2a assay using 10μM ATP and 100nM CK-2 peptide. Binding solution: 11% A, 80% B, 9% 5M NaCl, 1:2400 binding reagent.



Conclusions

- The new IMAP Progressive Binding system expands the range of application of IMAP assays to include substrates of high acidic character.
- The new system allows optimization of the assay to include much higher ATP concentrations. In some cases up to 1 mM ATP may be used.
- Increased stability of the working solutions is attained.