Fluorescence Polarization Assays of Proteases with The IMAP Platform

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Abstract

The IMAP® fluorescence polarization (FP) assay platform is a homogeneous, non-antibody-based system applicable to a wide variety of protein kinases. In this assay, fluorescently-labeled phosphopeptides are captured on modified nanoparticles through interactions with immobilized trivalent metals, resulting in high polarization values. We have now developed a generic, sensitive and efficient protease assay that uses this same IMAP platform. Several of these IMAP protease assays are demonstrated. The FP assay system has a distinct advantage over other platforms, because it is homogeneous, non-radioactive, and sensitive. In addition, the IMAP protease assays use peptide substrates that incorporate a cognate sequence including the scissile peptide bond. Such substrates provide the closest similarity to the cognate protein substrate and should therefore better predict compound potency.

Principle of the IMAP Protease Assay

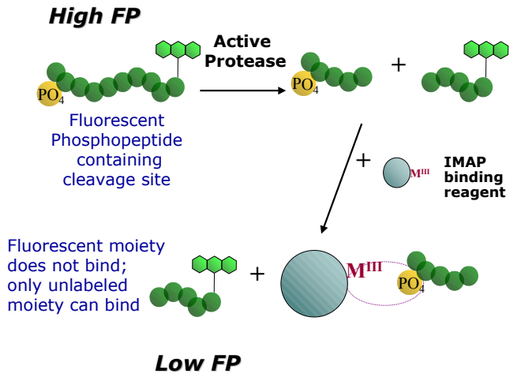


Fig 1. The IMAP technology is based on the high affinity binding of phosphate by immobilized metal (M^{III}) coordination complexes on nanoparticles. This IMAP Binding Reagent complexes with phosphate groups on phosphopeptides. Such binding causes a change in the rate of the molecular motion of the peptide, and results in a high FP value observed for the fluorescent label attached at the end of the peptide. This fluorescently labeled phosphopeptide can be specifically cleaved by the protease. Protease cleavage will remove the phosphopeptide moiety from the fluorescent substrate, resulting in low FP.

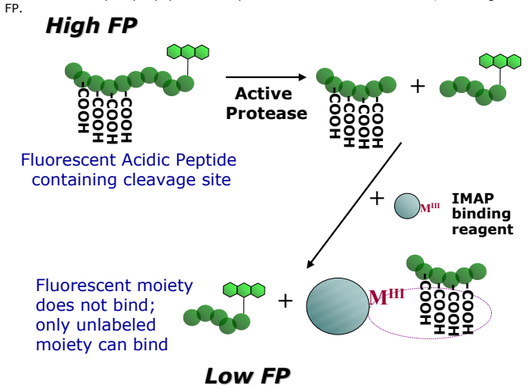


Fig 2. The IMAP Binding Reagent also complexes with the free -COOH groups on acidic amino acid residues. This interaction, although much weaker than the interaction with phosphate, does result in a stable high FP as long as four or more acidic residues are grouped on the peptide. An active protease will cleave at a point proximal to the fluorescent label, removing the acidic residues and resulting in low FP.

IMAP Protease Assay Set Up

For 384-well plate format
(or 1536-well plate format)

10 µL (1 µL) enzyme/test cmpnd
10 µL (1 µL) fluorescent substrate

5'-40' at RT

Add
60 µL (6 µL)
Binding Solution

30min-1h

Read FP

- **Substrate concentrations 10-1000 nM**
- **96 to 384 to 1536 well formats; low volume plates can be used**
- **Flexible reaction:Binding Solution ratio to fit any screen**
- **Can use both Original and Progressive IMAP Binding Systems**

Fig 3. The IMAP assay system is homogeneous and easy to use. All data shown is with the 384 well format. All fluorescently labeled peptides and phosphopeptides were synthesized by American Peptide Corp. All enzyme activity shown is expressed in U/mL, where 1 U = hydrolysis of 1 µmole of substrate/minute.

For each IMAP assay, the Analyst HT filter set was changed depending on the substrate label used. The Fluorescein method (485nm-20fwhm excitation, 530-25 emission, 505 nm dichroic) was used to obtain the FAM data; the Rhodamine method (530nm-25 fwhm excitation, 590nm-20 fwhm emission, 561 nm dichroic) was used for TAMRA.

IMAP Protease Assay: Cathepsin B

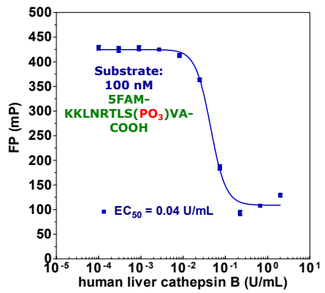


Fig 4. Human liver Cathepsin B (Calbiochem) titration in 20 mM NaOAc, 200 mM NaCl, 0.01% Tween20, 2mM DTT, pH 5.0 reaction buffer with 100nM 5FAM-KKLNRTL-pS-VA-COOH phosphopeptide in a 40 min reaction. Reactions were stopped by addition of 1/400 Progressive Binding Reagent in 100% IMAP Progressive Binding Buffer A. Each point is average of 4 replicates. 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 2-6 mP, and the Z' factor was >0.51 at concentrations greater than 0.02 U/mL of protease.

IMAP Protease Assay: Trypsin with Different Substrates

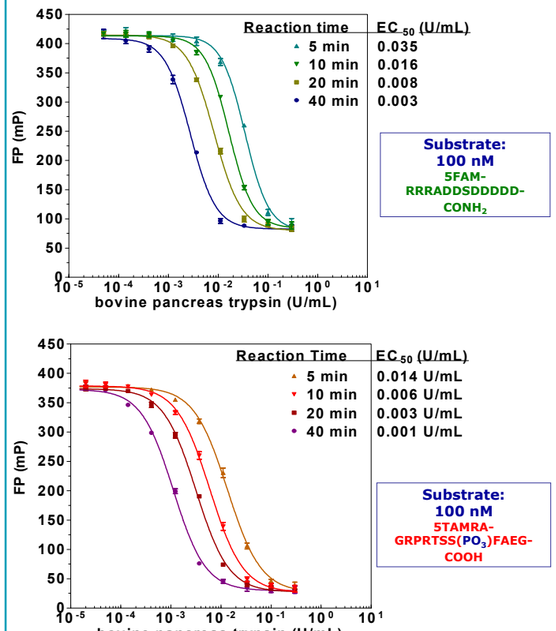


Fig 5. Trypsin from bovine pancreas (Calbiochem) was titrated with 100 nM 5FAM-CK2tide substrate (5FAM-RRRADDSDDDDD-CONH2) or 100 nM 5TAMRA-phospho-Crosstide (5TAMRA-GRPRTS-pS-FAEG-COOH) in pH8.8 reaction buffer (10 mM TrisHCL, 10 mM $MgCl_2$, 0.1% BSA, 0.05% NaN_3, 1 mM DTT, pH8.8). The Progressive binding solution contained 100% Binding Buffer A, 1/400 Binding Reagent. Binding incubation time was 1h. Curves were read with the Analyst 485/505/530 filter set (top graph) or 530/561/590 filter set (bottom graph). The error bars indicate the mP standard deviations of 4 replicates. The FP SDs ranged from 1-8 mP, and the Z' factor was >0.73 at all EC_{50} concentrations.

IMAP Protease Assay: Papain

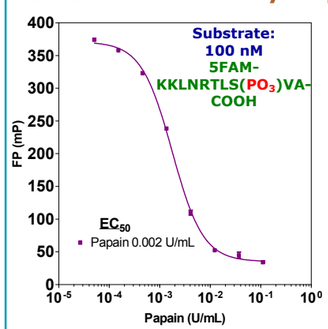
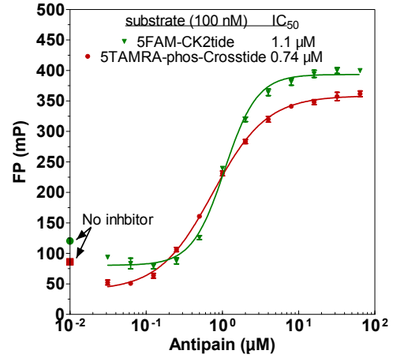


Fig 6. Top graph shows Papain (Sigma) titration in 50 mM MES, 250 mM NaCl, 0.05% BSA, 1mM DTT, pH 5.5 reaction buffer with 100nM 5FAM-KKLNRTL-pS-VA-COOH phosphopeptide. IMAP Original Binding Reagent (1/400) in Binding Buffer was added to each well after a 20 min reaction. Each point is average of 4 replicates. 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 1-5 mP, and the Z' factor was >0.75 at concentrations > 0.0005 U/mL.

IMAP Protease Assay: Trypsin Inhibition with Antipain



Green curve assay conditions:
100nM 5FAM-RRRADDSDDDDD-CONH2
0.014 U/mL Trypsin
20' reaction

Red curve assay conditions:
100nM 5TAMRA-GRPRTSS(PO3)FAEG-COOH
0.006 U/mL Trypsin
20' reaction

Fig 7. Green curve: Antipain (Calbiochem) was titrated in the presence of 0.014 U/mL Trypsin from bovine pancreas (Calbiochem) and 100 nM 5FAM-CK2tide substrate (5FAM-RRRADDSDDDDD-CONH2). Red curve: Antipain titration with 0.006 U/mL Trypsin and 100 nM 5TAMRA-phospho-Crosstide (5TAMRA-GRPRTS-pS-FAEG-COOH). For both inhibition curves, the reaction buffer was 10 mM TrisHCL, 10 mM $MgCl_2$, 0.1% BSA, 0.05% NaN_3, 1 mM DTT, pH8.8, and the reaction time was 20 minutes. The binding solution used was 100% Progressive Binding Buffer A, 1/400 Progressive Binding Reagent. Binding incubation time was 1h. Curves were read with the Analyst 485/505/530 filter set (green curve) or 530/561/590 filter set (red curve). Reactions with diluent in place of inhibitor are shown as single points. The error bars indicate the mP standard deviations of 4 replicates. The FP SDs ranged from 3-7 mP, and the Z' factor was >0.9 for the reactions without inhibitor.

The published Antipain IC_{50} value is 1-100 µM, which is comparable to the IC_{50} obtained in this IMAP assay. (Reference: Lyupina, Y.V., et al. 1996. *Eur. J. Pharmacol.* **304,** 23; Umezawa, H. 1976. *Methods Enzymol.* **55,** 678).

Advantages of IMAP Protease Assays

- **<u>Generic</u>: any specificity substrate, any reaction buffer, any protease assayed**
- **<u>Fast and sensitive</u> assay conserves time and reagents**
- **<u>Less substrate</u> per test point (≤ 100 nM peptide)**
- **<u>Choice of fluorescent labels,</u> choice of phosphopeptides or acidic peptides**
- **<u>IMAP IC_{50}s correlate well</u> with published values**
- **<u>Room temperature</u> screening**
- **<u>More physiological substrate</u> provides cognate sequence including the scissile peptide bond**

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