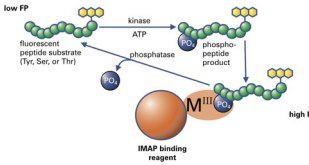
Use of IMAP TR-FRET detection mode for IMAP Substrate Finders

Joyce Itatani, Rich Sportsman and Annegret Boge
MDS Analytical Technologies, Sunnyvale, CA, USA.

Abstract

The IMAP 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. IMAP is offered in FP as well as in TR-FRET detection mode. Use of IMAP with fluorescently labeled peptides is now well established and has been tested on more than 100 kinases throughout the kinome. The IMAP Substrate Finder series of products is the key tool to identify suitable peptide substrates for novel kinases with the IMAP platform. So far the use of these tools has only been described in FP detection mode. We describe here application of the IMAP TR-FRET detection mode to the IMAP Substrate Finders.

IMAP FP assay



IMAP TR-FRET assay

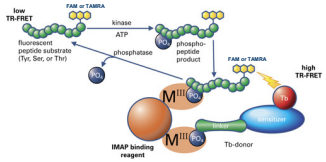


Figure1: Assay principle for IMAP TR-FRET and IMAP FP assay

Introduction

Identification of a suitable substrate for known or novel kinases has proven itself to be one of the bottlenecks in kinase screening assay development. The three Substrate Finders Molecular Devices offers for Ser/Thror Tyr kinases in their IMAP platform have proven themselves to be valuable tools to this end. With the introduction of the TR-FRET detection mode for IMAP, the need to also run these tools in TR-FRET mode has arisen. We show how we adapted the current Substrate Finder 2 for Serine and Threonine (R8140) kinases to TR-FRET detection and compare FP and TR-FRET detection for ERK2 and CDK2/CyclinA.

Optimization of the Substrate Finder protocol for TR-FRET

Substrate Finder peptides have been grouped by Binding Solution requirements with the goal of minimizing the number of Binding Solutions needed per plate. For use of TR-FRET detection, we chose three TR-FRET Binding Buffer conditions per original FP condition and ran the plate against a high concentration of PKA (data not shown). Based on these data we chose the TR-FRET Binding Buffer conditions to replace the FP Binding Buffer conditions indicated in bold in **Table 1**.

	FP conditions	TR-FRET conditions tested
1	**85%A, 15% B 1:400**	80%A, 20%B 1:600 **70%A, 30%B 1:600** 60%A, 40%B 1:600
2	**60%A, 40%B 1:1200**	**70%A, 30%B 1:800** 50%A, 50%B 1:800 30%A, 70%B 1:800
3	**40%A, 60%B 1:1200**	30%A, 70%B 1:800 **20%A, 80%B 1:800** 100%B 1:800
4	**25%A, 75%B 1:1500**	**100%B 1:1000**
5	**100%B 1:1750**	140%B 1:1000 160%B 1:1000

Table 1: Binding Buffer conditions tested to adapt the Substrate Finder to TR-FRET detection (chosen ones in bold). All TR-FRET Binding Buffer conditions included Tb Donor at a dilution of 1:400.

IMAP Substrate Finder protocol scheme

The peptides were reconstituted with 100 µM ATP in complete reaction Buffer. The assay was performed in presence and absence of enzyme for each substrate as described in the product insert. The appropriate Binding Buffer solutions were added and FP or TR-FRET read after o/n incubation at room Temperature.

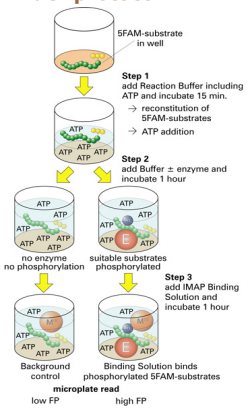


Figure 2: Substrate Finder Assay protocol

Substrate Finder Results for ERK2

The Substrate Finder for Ser/Thr 2 was originally developed for FP detection. Using the example of ERK2 we show here that similar hits for potential substrates are identified using either FP as originally recommended or TR-FRET detection mode after Binding Solution optimization as shown in **Table 1**.

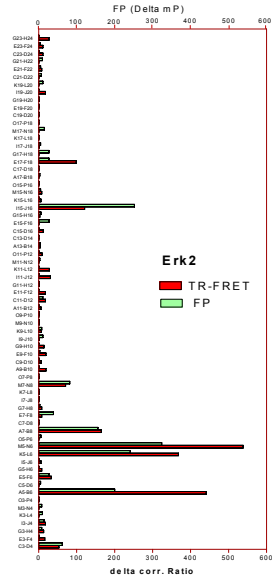


Figure 3: ERK2 (Upstate) was run at 0.5U/mL on the IMAP Substrate Finder for Ser/Thr 2 in the presence of 100µM ATP using Binding Buffers as described in **Table 1** (bold for TR-FRET).

Position	Sequence	FP ΔmP	TR-FRET Δcorr. Ratio
A5-B6	5FAM-YSPTSPSYSPTSPSYSPTSPS-OH	200	441
K5-L6	5FAM-TGPLSPGPF-OH	241	368
M5-N6	5FAM-ATGPLSPGPFGRR-OH	324	538
A7-B8	5FAM-APRTPGGRR-OH	156	165
I15-J16	5FAM-IPTTPITTTYFFFK-NH2	252	121

Table 2: Substrate Finder hits position and sequence for FP and TR-FRET detection on ERK2.

Substrate Finder Results for CDK2/Cyclin A

As a second example for the fidelity of TR-FRET detection on the Substrate Finder for Ser/Thr kinases 2 we chose CDK2/Cyclin A. Again the hits identified in FP detection mode match well with the hits identified in TR-FRET detection mode.

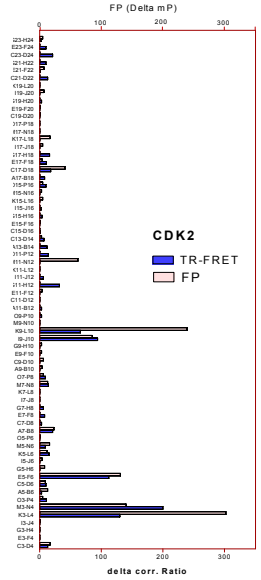


Figure 4: CDK2/CyclinA (Upstate) was run at 0.1U/mL on the IMAP Substrate Finder for Ser/Thr 2 in the presence of 100µM ATP using Binding Buffers as described in **Table 1** (bold for TR-FRET).

Position	Sequence	FP ΔmP	TR-FRET Δcorr. Ratio
K3-L4	5FAM-GGGPATPKKAKKL-OH	302	130
M3-N4	5FAM-GGGRSPGRRRRK-OH	140	200
E5-F6	5FAM-RRRFRPASPLRGPPK-OH	131	112
I9-J10	5FAM-FLAKSFGSPNRAYKK-OH	85	94
K9-L10	5FAM-PKTPKKAKKL-OH	239	66

Table 3: Substrate Finder hits position and sequence for FP and TR-FRET detection on CDK2/CyclinA.

Further evaluation of hits from Substrate finder results in FP and TR-FRET

A: ERK2

Dilution curves for three of the positive hits were run in FP (100 nM substrate)and TR-FRET detection mode (100 and 300 nM substrate). While the total EC_{50} did not change much between FP and TR-FRET detection, the latter resulted in lower standard deviations resulting in the option to run at lower enzyme concentrations. For all of the substrates a significant increase in assay window with increased substrate concentration could be observed as expected for TR-FRET.

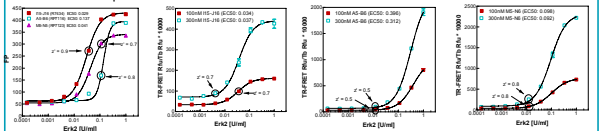


Figure 5: Dilution curves for selected hits from the Substrate Finder run on ERK2, circled data points indicate lowest concentration resulting in a z'-factor greater than 0.5. Panel A: FP detection, Panel B-D: TR-FRET detection.

B: CDK2/CyclinA

Also for CDK2/Cyclin A dilution curves for three example hits from the Substrate Finder were run in FP and TR-FRET detection mode as described for ERK2 with similar results.

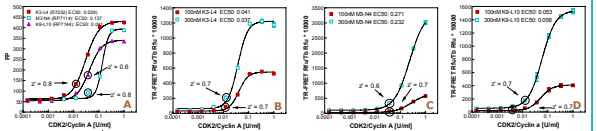


Figure 6: Dilution curves for selected hits from the Substrate Finder run on ERK2, circled data points indicate lowest concentration resulting in a z'-factor greater than 0.5. A: FP detection, Panel B-D: TR-FRET detection.

Summary

- Identification of suitable kinase substrates is one of the major bottlenecks in kinase assay development.
- The IMAP Substrate Finders, totaling more than 150 peptides in three different plates, can shorten this process significantly.
- IMAP offers two detection modes: FP and TR-FRET both are suitable to run the substrate finders as shown in this poster for the Substrate Finder for Ser/Thr kinases resulting in comparable results.
- The Substrate Finders are an integral part of the IMAP platform for kinase screening.

Molecular Devices