Use of IMAP® TR-FRET detection mode for the IMAP® AGC and CAMK Substrate Finder

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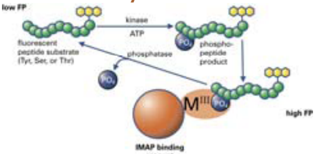
Abstract

One of the main bottlenecks in the development of in-vitro kinase assays is the identification of suitable peptide substrates for new kinases and their subsequent optimization for HTS performance. To this end, Molecular Devices has developed a number of Substrate Finder plates to run with its IMAP® platform. The IMAP® Technology utilizes the high affinity binding of phosphate to immobilized trivalent metals with two detection modes: TR-FRET and FP. We have previously described the equivalency of the assay results between TR-FRET and FP modes for the CMGC, CK1, STE, and TKL group Substrate Finder plate. Here we show this equivalency for the AGC and CAMK group Substrate Finder plate.

IMAP® FP assay



IMAP® TR-FRET assay

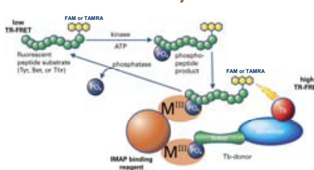


Figure1: Assay principle for IMAP® TR-FRET and IMAP® FP assay

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. Molecular Devices offers three Substrate Finders for Ser/Thr or Tyr kinases in their IMAP® platform which have proven themselves to be valuable tools to this end. With the introduction of the IMAP® TR-FRET detection, the need to also run these tools in TR-FRET mode has arisen. Here we show how we adapted the Ser/Thr 1 (CamK/AGC group) Substrate Finder Kit (R8131) to TR-FRET detection and compared FP and TR-FRET detection for Pim1 and PKA kinases.

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. 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
1	100%A 1:400	**80%A, 20%B 1:600** 70%A, 30%B 1:600 60%A, 40%B 1:600
2	75%A, 25%B 1:600	**50%A, 50%B 1:600** 40%A, 60%B 1:800 30%A, 70%B 1:800
3	60%A, 40%B 1:600	**50%A, 50%B 1:600** 40%A, 60%B 1:800 30%A, 70%B 1:800

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 values were read after overnight incubation at room temperature

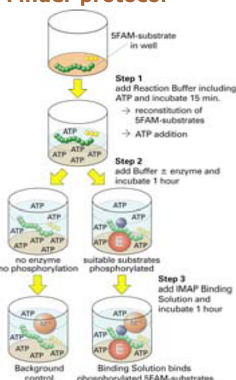


Figure 2: **Substrate Finder Assay protocol**

Substrate Finder Results for Pim1

The Substrate Finder for Ser/Thr kinases 1 (R8131) was originally developed for FP detection. Using the example of Pim1 we show here that similar hits for potential substrates are identified using either FP or TR-FRET detection mode after Binding Solution optimization as shown in Table 1.

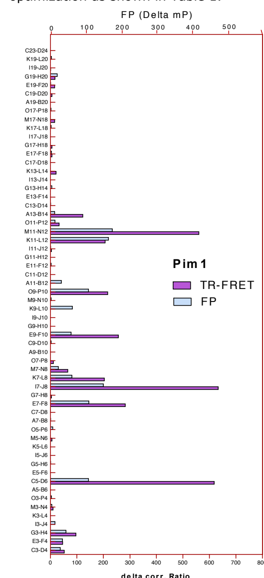


Figure 3: Pim1 (Upstate) was run at 0.5U/mL on the IMAP® Substrate Finder for Ser/Thr 1 (R8131) in the presence of 100µM ATP using Binding Buffers as described in Table 1 (bold for TR-FRET).

Position	Sequence	FP △mP	TR-FRET △corr. Ratio
M11-N12	5FAM-AKRRRLSSLRA-OH	233	560
K11-L12	5FAM-ERMRPRKRQGSVRRRV-NH2	219	206
I7-J8	5FAM-RKRRQTSM-OH	200	633

Table 2: Substrate Finder hits position and sequence for FP and TR-FRET detection on Pim1.

Substrate Finder Results for PKA

As a second example for the fidelity of TR-FRET detection on the Substrate Finder for Ser/Thr kinases 1 (R8131), we chose PKA. Again the hits identified in FP detection mode match well with the hits identified in TR-FRET detection mode.

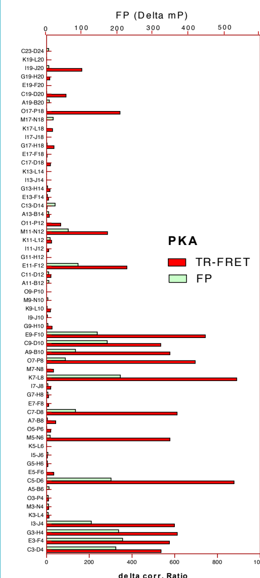


Figure 4: PKA (Upstate) was run at 0.5U/mL on the IMAP® Substrate Finder for Ser/Thr 1 (R8131) in the presence of 100µM ATP using Binding Buffers as described in Table 1 (bold for TR-FRET).

Position	Sequence	FP △mP	TR-FRET △corr. Ratio
K7-L8	5FAM-GRTGRRNSI-NH2	345	890
C5-D6	5FAM-KKRPQRRYSNVF-OH	302	877
C3-D4	5FAM-LKKLTRRPSFSAQ-OH	325	536

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

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

A: Pim1

Dilution curves for three of the positive Pim1 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.

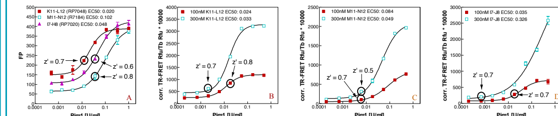


Figure 5: Dilution curves for selected hits from the Substrate Finder for Pim1 kinase. Circled data points indicate lowest concentration resulting in a z'-factor greater than 0.5. Panel A: FP detection, Panel B-D: TR-FRET detection.

B: PKA

Dilution curves for three of the positive PKA hits were run in FP and TR-FRET detection mode as described for Pim1 with similar results.

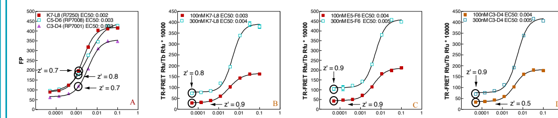


Figure 6: Dilution curves for selected hits from the Substrate Finder for PKA kinase. Circled data points indicate lowest concentration resulting in a z'-factor greater than 0.5. A: FP detection, Panel B-D: TR-FRET detection

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.
- The IMAP® Technology offers two detection modes, FP and TR-FRET, both of which produce equivalent results as shown in this poster for the Ser/Thr 1 (CamK/AGC group) Substrate Finder kit (R8131).
- The Substrate Finders are an integral part of the IMAP® platform for Kinase screening.

Molecular Devices