FP and TR-FRET kinase assays with automated liquid handling

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Introduction

Protein kinases are central to the regulation of many cellular processes. In recent years they have emerged as one of the most important classes of drug targets for cancer and many other diseases. Here, the kinase Blk (B lymphoid tyrosine kinase), a member of the Src family of protein tyrosine kinases involved in B cell differentiation and proliferation, was assayed using IMAP®, a non-antibody-dependent FP method. IMAP technology enables rapid, non-radioactive assay of a wide array of kinases and is suited to both assay development and high-throughput screening.

As screening laboratories seek to boost their throughput, the demand for automation of assay setup increases. We explored the feasibility of using an automated liquid handling system, the AquaMax® DW4, to assemble IMAP kinase assays and demonstrate that these results compare favorably to those obtained with hand-pipetted assays. Z' factors were well above 0.5, and EC_{50} values for Blk were comparable to published values. Staurosporine curves yielded IC_{50} values similar to those already published. These results demonstrate that automation of assay setup, including dispensing of various reagents and volumes, facilitates higher throughput without sacrificing accurate results.

Materials and Methods

Blk kinase assays were performed using IMAP FP or TR-FRET Progressive Binding System with Tween, and FAM-Blk/Lyntide substrate. Staurosporine, a known inhibitor of Blk, was assayed at concentrations ranging from 0.5 nM to 3 µM. Calibration curves using known mixtures of phosphorylated and non-phosphorylated FAM-Blk/Lyntide were also generated.

Kinase assay setup
5 µL 4x staurosporine in complete reaction buffer
5 µL 4x Blk kinase in complete reaction buffer
10 µL 2x ATP + 2x FAM-Blk/Lyntide substrate

Calibrators
20 µL 0%-100% phosphorylated FAM-Blk/Lyntide

FP Binding Solution
60% Buffer A
40% Buffer B
1:1200 Binding Reagent

TR-FRET Binding Solution
35% Buffer A
65% Buffer B
1:800 Binding Reagent
1:400 Tb Donor

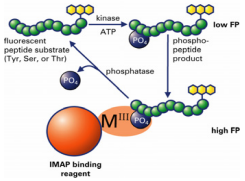
In DW4-dispensed assays, the staurosporine dilution series were manually pipetted into assay plates, then the kinase and ATP/substrate reagents were dispensed automatically using a DW4 with a 384-well dispense head. For calibration curves, calibrators were manually pipetted into assay wells. Following a one-hour kinase assay incubation, 60 µL IMAP Binding Solution was dispensed per assay well using the DW4. In hand-pipetted assays, all reagents were pipetted using multi-channel manual pipettors.

After a 2-hour (FP) or 16-hour (TR-FRET) incubation with Binding Solution, all plates were read on a SpectraMax® M5 multi-detection microplate reader, and data were analyzed using SoftMax Pro® software.

IMAP assay principle

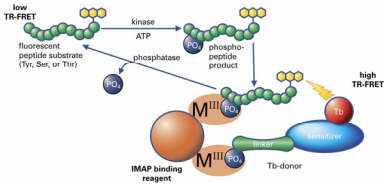
Fluorescence polarization

Figure 1. IMAP is a homogeneous assay technology based on binding of phosphate through immobilized metal coordination complexes on nanoparticles (IMAP binding reagent). In FP assays, when phosphorylated substrate binds to binding reagent, molecular motion of the peptide is altered and fluorescence polarization for the fluorescent label attached to the peptide increases.



TR-FRET

Figure 2. In IMAP TR-FRET assays, an additional reagent, the Tb Donor, binds to binding reagent, enabling FRET to occur between Tb and fluorescent label on the peptide.



Results: Kinase inhibition assays

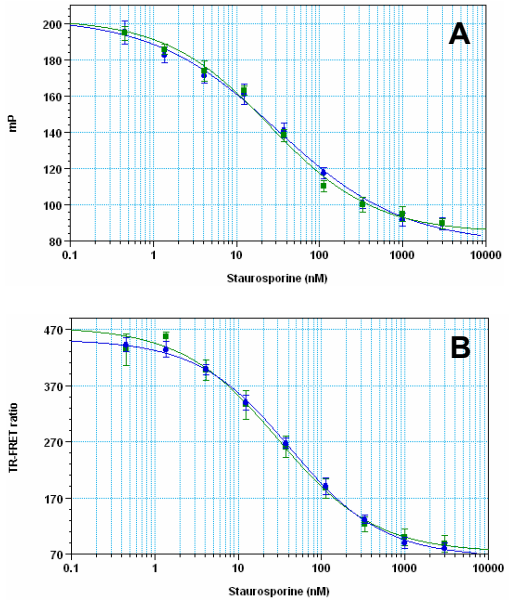


Figure 3. Staurosporine inhibition curves. Blk at 80% maximal activity was assayed for staurosporine concentrations ranging from 0.5 nM to 3 µM. IMAP FP (A) or TR-FRET (B) assays dispensed manually (blue circles) or using DW4 (green squares). In FP assays, IC_{50} values for staurosporine were 31 nM for manually pipetted and 25 nM for DW4-dispensed. In TR-FRET assays, IC50 values were 42 nM (manual) and 30 nM (DW4). Z' factors ranged from 0.73-0.81.

Results: Calibration curves

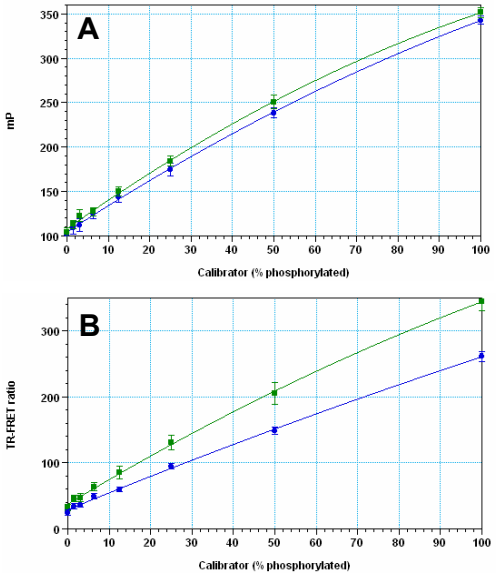
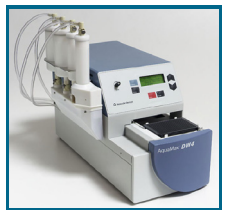


Figure 3. Calibration curves with 0% to 100% phosphorylated FAM-Blk/Lyntide. IMAP FP (A) or TR-FRET (B) assays dispensed manually (blue circles) or using DW4 (green squares).

Dispensed using AquaMax DW4			
% phosphorylated	Average mP	Std dev	CV%
100	351.5	5.50	1.6
50	250.8	7.58	3.0
25	184.3	5.35	2.9
12.5	150.1	5.14	3.4
6.3	127.3	4.62	3.6
3.1	122.9	6.32	5.1
1.6	113.0	4.73	4.2
0	104.0	5.51	5.3

Dispensed manually			
% phosphorylated	Average mP	Std dev	CV%
100	342.8	4.76	1.4
50	238.6	5.38	2.3
25	175.0	7.21	4.1
12.5	144.1	5.95	4.1
6.3	125.5	6.14	4.9
3.1	112.3	7.60	6.8
1.6	109.1	7.56	6.9
0.0	102.4	7.06	6.9

Table 1. Data for DW4 and manually dispensed FP calibration curves. Assay windows are nearly identical, and all CV% values are below 7.



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