

Microfluidic Kinase Selectivity Screening Assays

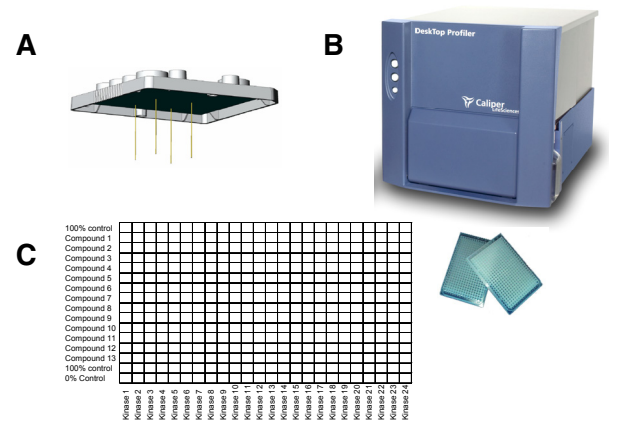
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Abstract

A plug and play panel of 48 kinase assays has been developed using Caliper's off-chip mobility shift assay. The selected kinases include members from all of the major kinase families and are broadly distributed throughout the kinome. The set consists of individual kinases pre-dispensed one per column in 384 well plates and matching substrate plates containing fluorescein-labeled peptidic substrates with ATP at its apparent Km value. The simplified work flow allows a user to thaw plates, dispense reconstitution buffer and compounds in DMSO into the enzyme plate, and then transfer the substrate to start the reactions. All operations can be done manually or using liquid handlers. After a 90 minute incubation, reactions are quenched and analyzed. The extent of phosphorylation is measured by electrophoretic separation of the product and substrate on Caliper's DeskTop Profiler™ platform. Storage at -80 °C preserves the enzyme and substrate activity for >4 months. Dose response curves for a set of commercially available kinase inhibitors shows that the assays are robust and reproducible. The observed pattern of activity of the inhibitors against the enzyme panel underscores the usefulness of selectivity screening early in the development of lead compounds

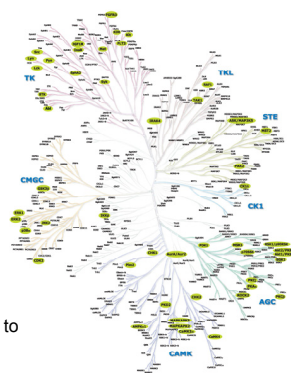
Plug and Play Kinase Selectivity Profiling System

The system consists of **A**) the ProfilerPro™ microfluidic chip for mobility shift assays, **B**) DeskTop Profiler reader and **C**) ProfilerPro plates. Two plates contain a total of 48 Kinases (24 per 384 well plate) pre-dispensed in columns. The plates are stored at -80 °C until ready to use.



Procedure:

- 1) Thaw enzyme and substrate plates.
- 2) Add compounds in DMSO to the common reconstitution buffer. (13 compounds + pos and neg controls)
- 3) Dispense 16ul/well of each compound in reconstitution buffer to each row of the plate to reconstitute the kinases.
- 4) Pre-incubate the compounds with the enzymes for 15 minutes.
- 5) Add 10 μL/well of the substrate/ATP plate to initiate the reactions.
- 6) Incubate at 25 °C for 90 minutes.
- 7) Add termination buffer to quench the reactions.
- 8) Read plate on DeskTop Profiler.



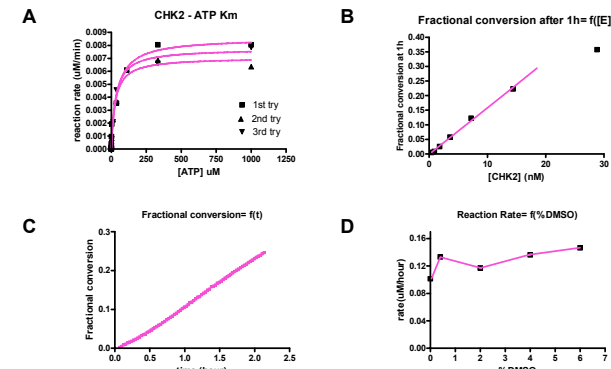
The 48 kinases in the two plates are broadly distributed throughout the kinome.

Kinase Selectivity Panel Assay Conditions

- All assays were run at ATP/Km = 1 for each enzyme
- Common buffers were used and cofactors added as required:

Reaction Buffer:
100 mM HEPES, pH = 7.5
10 mM MgCl₂ or MnCl₂
0.003% Brij-35
1 mM DTT
4% DMSO
[E] = 0.025 to 30nM with mean = 7.2 nM
[S] = 1.5 μM (<<K_m peptide)

Separation Buffer:
100 mM HEPES, pH = 7.5
0.015% Brij-35
1 mM EDTA
0.1 % Coating Reagent 3
5% DMSO

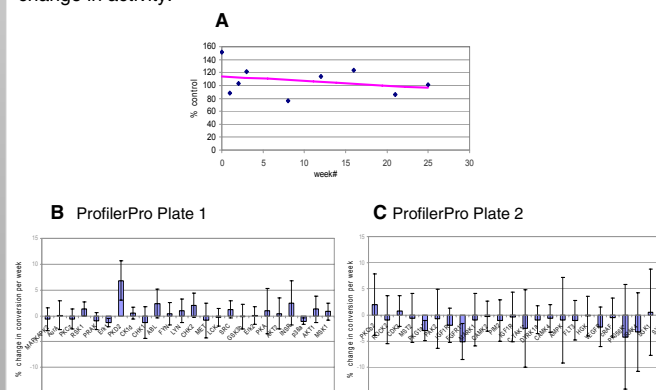


Sample assay development data for CHK2 using 1.5 μM 5-FAM-KKKVSRGLYRSPMPENLNRR-COOH peptide showing **A**) ATP K_{m,app} of 57.8 ± 10 μM, **B**) reaction linearity as a function of [E], **C**) reaction linearity with time and **D**) Effect of DMSO (up to 6%) on reaction rate.

Low volumes of low concentration enzymes can be stored frozen for long periods without significant degradation

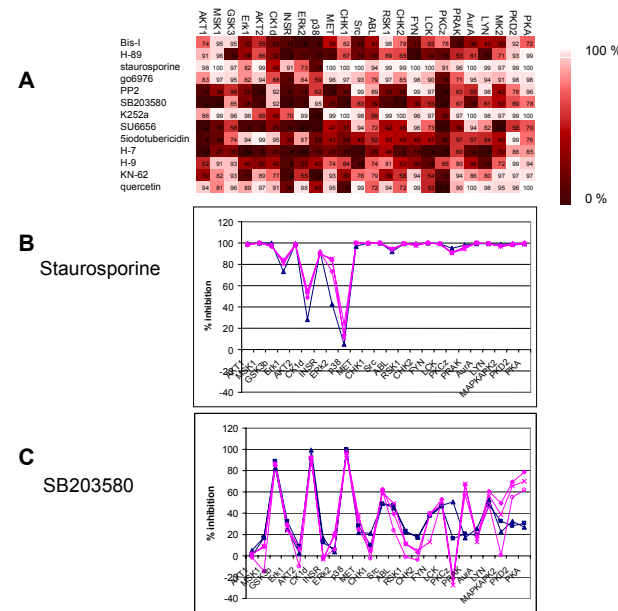
Measurement of enzyme inactivation. Enzymes were dispensed onto 384 well plates in a proprietary storage buffer, frozen and stored at -80°C. Enzyme activity was measured over several months by: thawing, adding 16 μL/well of reconstitution buffer, adding 10 μL/well of substrate/ATP, incubating at 25°C and quenching with termination buffer. Analysis was performed on a Caliper LabChip 3000.

Stability data. **A.** sample data shown for PKCz over 25 weeks. **B/C** The percentage of change in conversion per week is shown for the 48 kinases (ProfilerPro plates 1 and 2), with error bars representing 95% confidence intervals. **B.** ProfilerPro plate 1 study was run for 25 weeks. **C.** ProfilerPro plate 2 stability study was run for 12 weeks (*indicates data for 7 weeks only). Generally, no enzyme showed a statistically significant change in activity.



Kinase Selectivity Screening

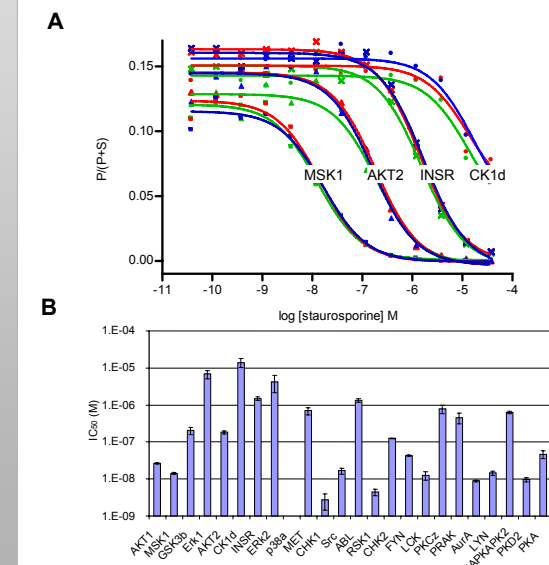
(A) A set of 13 commercially available kinase inhibitors was run at a concentration of 10 μM against the first ProfilerPro plate. Selectivity data for staurosporine **(B)** and SB203580 **(C)** run at Caliper (—) and at an external site (—) showed good agreement.



IC₅₀ determinations

The potency of a series of commercially available kinase inhibitors was tested against ProfilerPro plate 1.

A) Sample replicate IC₅₀ data for staurosporine. **B)** Staurosporine IC₅₀ data for plate 1 (mean +/- SD) shows low variability.

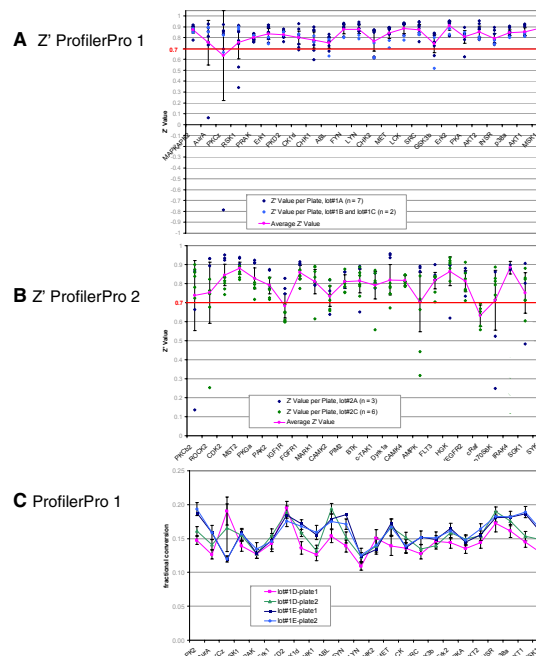


Reproducibility

Z' values were determined over several lots for **(A)** ProfilerPro Plate 1 and **(B)** ProfilerPro Plate 2. **C.** Conversion variability over 2 lots, 2 plates each.

$$Z' = 1 - \frac{3\sigma_{c_1} + 3\sigma_{c_2}}{|\mu_{c_1} - \mu_{c_2}|}$$

with error bars representing 95% confidence.



Summary

- Kinases could be pre-dispensed onto plates, frozen and stored for > 4 months without significant changes in activity.
- Assays for 48 broadly distributed kinases were developed with common reaction conditions.
- Optimized microfluidic assays resulted in very reproducible data.
- Measured inhibition at 10 μM compound and IC₅₀'s agreed among different sites.
- The ProfilerPro plates and the DeskTop Profiler constitute an effective means of performing in-house kinase selectivity profiling.