

Evaluation of the Nanostream μ PLC for kinase assay screening

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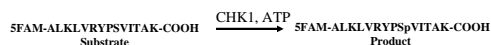
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Abstract

The objective of this work was to evaluate the performance of the micro parallel liquid chromatography (μ PLC) system from Nanostream as a separation-based assay platform for small molecule secondary screening on kinase drug targets. A Chk1 enzymatic assay in which we analyzed the conversion of a fluorescent-labeled peptide substrate to a phosphorylated product was chosen for this study. DNA damage activates checkpoint pathways that cause cell cycle arrest, providing cells time for DNA repair. Chk1 plays a critical role in activating these checkpoints, and inhibitors of this kinase inhibit cell cycle arrest and thereby lead to premature mitotic entry and cell death. The Chk1 kinase enzymatic reaction was performed in 384-well plates then transferred to the μ PLC system for separation analysis. The effect of 460 previously identified Chk1 small molecule inhibitors was evaluated in this assay. The potencies obtained with μ PLC was compared to values obtained in a 1536-well based time resolved fluorescence resonance energy transfer (TR-FRET) assay and a 1536-well based Immobilized Metal Assay for Phospho-Chemicals (IMAP). The advantage of the HPLC over the other assays is that since it is a separation-based assay there is reduced interference from compounds which are themselves fluorescent or which quench the fluorescence of the signal and our data reflects this. The μ PLC results were also highly reproducible with excellent statistic parameters (Z factor and %CV). Hence, the Nanostream μ PLC technology is a novel important addition to our HTS tools for kinase screening.

Assay principle



Assay protocol

The protocol for measuring the potency of Chk1 inhibitors in the Nanostream μ PLC assay was as follows:

- 20 μ l of Chk1 kinase (40nM) was dispensed into a non-binding 384-well plate.
- 1 μ l of test compound (2mM stock in DMSO) or DMSO was added to each well.
- To start the enzymatic reaction, a 20 μ l mixture of ATP (200 μ M) and FAM labeled-peptide substrate (1 μ M) was dispensed to each well.
- Incubate for 30 minutes at 25°C.
- Add 40 μ l of quench solution containing EDTA (50mM) into each well to stop the reaction.
- 1 μ l of the final reaction volume was injected into and read (fluorescence) on the Nanostream μ PLC system.

Calculations

Compound = compound assayed in well

Basal = Enzyme concentration to obtain 70% of substrate conversion into product, no compound in well

Background (Bkg) = no ATP, no compound in well

$$\% \text{ conversion} = 100 \times \frac{\text{Product}_{\text{Peak Area}}}{\text{Product}_{\text{Peak Area}} + \text{Substrate}_{\text{Peak Area}}}$$

* Peak areas calculated by Nanostream Analysis Software

$$\% \text{ Inhibition}_{\text{compound}} = \left(1 - \frac{\% \text{ conversion}_{\text{compound}} - \text{median}(\% \text{ conversion}_{\text{Bkg}})}{\text{median}(\% \text{ conversion}_{\text{Basal}}) - \text{median}(\% \text{ conversion}_{\text{Bkg}})} \right) \times 100$$

Nanostream LD μ PLC-FD Method and Materials



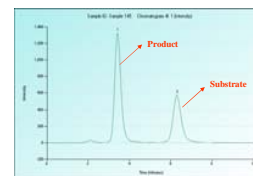
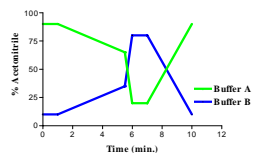
The system is equipped with binary LC pumps (Shimadzu) connected to dual 24-channel fluorescence and UV detectors. The 8-needle autosampler and plate stacker allows random access to wells of a 384-well plate in register of 8 (e.g., A1-8, C9-16, N17-24).

The 24-column microfluidic Brio cartridge enables multiple samples to be analyzed in parallel while reducing sample and solvent consumption. A variety of column lengths and stationary phases can be selected to fulfill throughput requirements and to optimize performance for specific experimental protocols.

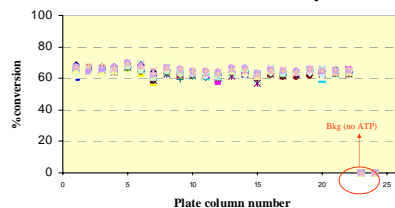
384 samples were assayed in ~3 hours using Nanostream LD system. Traditional single-column HPLC/UV-FD system with same sample count would have taken ~58 hours.

LD Parameters

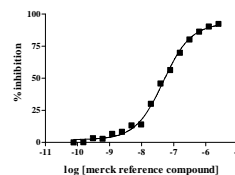
Injection Volume: 1 μ L.
Fluorescence Detection: Excitation Filter = 482 nm, 25 nm bandwidth and Emission Filter = 536 nm, 60 nm bandwidth.
Mobile Phase A: 25 mM NaH₂PO₄ – Na₂HPO₄ (pH 7.4)
Mobile Phase B: Acetonitrile



Assay validation

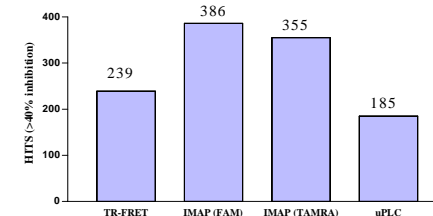


Scatter plot of DMSO plate: Statistical parameters are as follows: Median basal 64% conversion, Standard deviation=2.2%, %CV=3.4 and Z factor=0.9

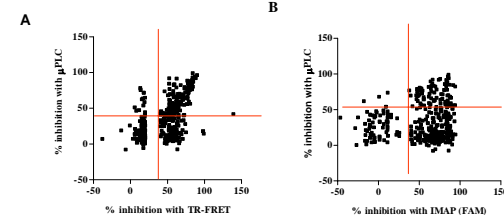


Dose response of a reference compound used to validate the μ PLC assay for CHK1. Enzymatic reaction were carried out on a 384-well plate, quenched, and separated on the μ PLC system. The calculated IC₅₀=47nM is similar to that obtained using TR-FRET (41nM), IMAP-FAM (85nM) and IMAP TAMRA (87nM)

Results



Comparison of the number of Chk1 small molecule antagonists identified across four assays.



Correlation plots for full length Chk1 pharmacology comparing μ PLC to HTRF (A) and IMAP (B).

Summary

- IC₅₀ values obtained using μ PLC technology were similar to those obtained using TR-FRET and IMAP technologies
- Fluorescence interference was not an issue because separation of the fluorescent quencher and fluorescent substrate was achieved.
- False positive hits from FP (~53%) and TR-FRET (~33%) technologies were identified.

References

- Lemmo, A.V., Hobbs, S., and Patel, P. "Micro parallel liquid chromatography: enabling technology for discovery analytical chemistry". *Assay Drug Dev. Tech.* 2 (2004) 389-395.
- Sylvie Jezequel-Sur et al. "Mixing two differently labeled substrates in one IMAP assay to improve data quality" *Anal. Biochem.* 360 (2007) 312-314