Introduction

Fragment-based lead discovery is establishing itself as a valuable approach for the discovery of novel small molecule leads. It relies on establishing a better understanding of the binding mechanism of a ligand for its binding partner by probing for “hot spots” on the protein target with small molecule fragments (typically <300 Daltons). Its simplicity is in the principle of building a pharmacophore from the ground up. Successful selection requires data on binding affinity as well as extensive SAR information provided by a variety of techniques including NMR, X-ray crystallography, SPR as well as other biophysical techniques. This information will ultimately yield valuable insights into the molecular recognition process that aid in the design of more selective and potent drugs with fewer off target effects.

Fragment-based screening is not without its challenges, for example NMR is a “gold standard” technique but is often used for detailed follow-up of initial hits selected in biochemical assays, due to its low throughput and high protein consumption. On the other hand biochemical assays can provide important initial insights into the binding affinities, but these small chemical fragments typically have binding affinities in the 50 µM to low millimolar range. Therefore, they must be screened at much higher concentrations to detect these weak binding interactions. This results in solubility problems as well as high false positive and negative rates due to compound interference when using fluorescence-based biochemical assays. In addition most biochemical assays provide limited information on the differences in the binding mechanisms of fragments.

This white paper describes the use of the microfluidic mobility shift assay as an alternative to traditional biochemical assays for fragment-based screening. In addition it has the capability to provide structural insights that compliment techniques like NMR.

Introduction to the Microfluidic Mobility Shift Assay

Caliper's Microfluidic Mobility Shift Assay (MMSA) technology is designed for in vitro enzyme assays. It combines the advantages of capillary electrophoresis and microfluidics providing direct measurement of substrate and product, rapid assay development and real time kinetic reads. The assay measures the changes in mobility of molecules that have been biochemically modified for example by phosphorylation, and deacetylation electrophoretically. As the reaction mixture that is sipped from a microplate well travels through the microfluidic channel containing an acrylamide gel the substrate and product are separated in a time resolved fashion. At the end of the channel an LED (470 nm) excites the fluorescent labeled molecules as they travel by the detection window and the amount of separated reaction components are measured (figure 1).
The MMSA provides a direct readout of the product conversion and substrate remaining, a direct measure of enzyme inhibition (or activation). There is no need for coupling reagents (antibodies) or secondary enzymes; this dramatically reduces the likelihood of losing valuable time and data due to assay artifacts. Since the technique is a separation based approach all the reaction components are identified (figure 2), unlike homogenous assays where the entire contents of the reaction are read simultaneously. This reduces the chances of false positives due to autofluorescence of the compound being screened, especially important when working at high concentrations typical of fragments.

The MMSA has several advantages for fragment-based screening of enzyme drug targets:

- Fewer false positives
- Improved assay reproducibility
- Direct measurement of enzyme activity
- Identification of novel fragment binding mechanisms

The technique has been utilized on a number of enzyme drug targets including protein kinases, lipid kinases, protein phosphatases, proteases, histone deacetylases, and sirtuins.

Direct Measurement of Enzyme Regulation

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During assay validation scientists from EMD Serono intentionally selected compounds that exhibited a high degree of autofluorescence to see how they would affect the MMSA assay performance, they discovered that of 321 compounds with known interference problems in biochemical assays only 21 were detected in the MMSA and of those none affected the assay results. They also observed that a further advantage of the MMSA is the techniques ability to normalize the data for well to well substrate dispensing variations. This is done by reporting the data as a product/substrate ration and not as absolute peak heights. This eliminates errors due to dispensing variations. In addition the calculated peak sum (sum of substrate and product peak heights) provides a QC tool for monitoring substrate dispensing precision.

In another study done by scientists at Novartis Institute for Biomedical Research in Basel, Switzerland several different biochemical assay platforms were assessed for their robustness to artifacts from fluorescence interference of fragments especially at high concentrations. It was determined that the MMSA was the most robust with respect to artifacts from autofluorescent and fluorescence quenching of fragments, (figure 3).

“MMSA technology appears to be immune to fluorescence interference” EMD Serono

“MSA should be the most robust method with respect to artifacts stemming from autofluorescent and fluorescence quenching fragments” Novartis

**Mode of Action**

One of the most important advantages of the MMSA is that it can give insights into mechanistic differences of inhibition of fragments on enzymes and cofactors. This is important because it may reveal unique features that can result in the discovery of novel compound scaffolds. The assay technique is not sensitive to ATP concentrations so for ATP dependent enzymes such as kinases it is possible to screen using a range of ATP concentrations and identify ATP competitive, non-competitive, and uncompetitive inhibition. In addition when used for IC50’s and in real time kinetic mode experiments can provide information that could normally remain unnoticed. This would include the effects of cofactors, covalent complex formation, slow on rates, and fragment insolubility.

Scientists at the Tokyo University of Agriculture and Technology used the MMSA to assess inhibitors of WNK1 kinase. Comparison of dose-dependent inhibition at different ATP concentrations indicated that inhibitors were ATP competitive. In addition they discovered compound precipitation from 60 to 100 µm, (figure 4).
I C\textsubscript{50} curves for Tyrphostin 47 show no inhibition at higher ATP concentrations indicating that the compound might be ATP competitive. In addition the compound exhibited only 60% inhibition at the maximum dose due to compound insolubility at concentrations greater than 60 µm.

Enzyme progress curves provide important information on the mechanism of inhibition which can lead to identification of novel inhibitors. Progress curves are easy to perform using the MMSA in the kinetics mode and consume less than .2 µL of reaction mixture for each time point. Work done at the Broad Institute with the modulation of histone deacetylase activity has lead to the discovery of both classical and slow-binding inhibitors for HDAC3 and HDAC6. This may explain the differences in HDAC inhibition observed in cell based assays\textsuperscript{5}, (figure 5).

Scientists at Pfizer were using fragment based screening to identify small molecule modulators of PDK1 kinase\textsuperscript{6}. They were looking for both ATP competitive and allosteric inhibitors, specifically ligands for the PIF pocket which is a binding pocket which recognizes non-catalytic motifs. It had been demonstrated that small molecules could modulate activity. The MMSA was employed for the purposes of screening fragment activity along with a Kinase-Glo assay. Results measuring fragments that modulate activity through the PIF site using the MMSA were in agreement with the NMR competition assay; however the Kinase-Glo assay was not able to detect these compounds due to that fact that the PIF pocket was blocked by the PDKtide substrate.

“Caliper assay data and \textsuperscript{19}F NMR assay data on the PIF pocket fragments and structurally related compounds identified them as potentially allosteric activators of PDK1 function” Pfizer\textsuperscript{6}
The ability to make real time observations provides benefits in assay development as well. For example work done at EMD Serono on developing a protein tyrosine kinase assay indicated that there was an increase in the initial rate of enzyme activity when a cofactor was added 5 minutes prior to the initiation of the reaction, as compared to when the cofactor was added at the initiation\(^2\), (figure 6).

![Figure 6](image)

**Figure 6.** Increase in initial reaction rate (blue) with addition of cofactor 5 minutes ahead of time as compared to when cofactor was added at initiation (red)

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**Summary**

LabChip | EZ Reader

The LabChip EZ Reader is a breakthrough benchtop instrument which facilitates kinase testing and other enzyme screening and profiling. When combined with ProfilerPro kits, a low cost, turn-key solution of lab-bench kinase panel profiling is provided. LabChip assays are separations-based, so the quality of results exceeds what is achievable in homogenous, well-based assays. The LabChip EZ Reader runs kinase and enzymatic assays with a 4-Sipper microfluidics chip.

Utilizing Caliper’s widely adopted mobility shift microfluidics assay technology, the LabChip EZ Reader analyzes kinase activity by ‘sipping’ stopped reactions from 96 or 384 well microtitre plates. Caliper’s Mobility Shift Assay technology is designed for in vitro enzyme assays aimed at the drug discovery process. It combines the advantages of capillary electrophoresis and microfluidics providing direct measurement of substrate and product, rapid assay development and real time kinetic reads.

The EZ Reader is the perfect platform for running kinase profiling with the Caliper ProfilerPro Kits. ProfilerPro Kinase Profiling kits consist of matched pairs of 384well kinase assay and plates complete with substrates. The assay plates each contain 24 different kinases, pre-dispensed and frozen. The substrate plates contain the appropriate fluorescent labeled peptides and ATP.

Using LabChip EZ Reader researchers can monitor enzyme reactions and kinase inhibitors over time, resulting in more detailed data resulting in using fewer reagents. The advantages of microfluidics technology with real time kinetic analysis include: simplified and accelerated assay development and optimization, calculation of \(K_m\) and \(K_i\) values from initial rates, and determination of enzyme linearity.
Reference:


