Assay Development and Inhibitor Characterization of Protein Kinases Using a Lab Chip 3000

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Materials and Methods

All data shown were collected using the Caliper LabChip 3000 instrument and analyzed using a software package, HTS Well Analyzer, designed by Caliper for use with the LabChip3000. All assays performed were introduced onto the Sipper Chip via capillaries (i.e. Off-Chip Mobility Shift assays) as described in Figure 1. See figure legends for specific experimental conditions.

Steady State Characterization Data for determining steady state kinetic parameters were fit to Equation 1, where v is velocity, Vmax is the maximum velocity, S is the substrate concentration, and Km is the Michaelis constant:

Equation 1 v = Vmax * S / (Km + S)

Mechanism of Action Data for determining the mechanism of inhibition were fit to Equations 2 and 3 (describing competitive and noncompetitive inhibition, respectively) where v is velocity, Vmax is the maximum velocity, S is the substrate concentration, Km is the Michaelis constant, I is the inhibitor concentration, and Ki is the inhibition constant:

Equation 2 v = Vmax * S / (Km * (1+I/Ki) + S)

Equation 3 v = (Vmax * S / (1 + I/Ki)) / (Km + S)

Inhibitor Kinetics The off-rate progress curves were fit to Equation 4, where p is product, *vs.* is the initial velocity, v is the final velocity, t is time, and kapp is the apparent first order rate constant (koff). The progress curves for irreversible inhibition were fit using Equation 5. Second order replots (kobs vs. inhibitor) were fitted to Equation 6.

Equation 4 p = vs*t +(v - vs)*(1-exp(-kapp*t))/kapp

Equation 5 pt = v0 * (1 - exp(-kobs * t))/kobs + p0

Equation 6 kobs = kinact * I / (Ki * (1+S/Km) + I)

Introduction

Protein kinases represent potential drug targets across several therapeutic areas at Pfizer. An assay technology that can provide both medicinal chemistry support and mechanism of inhibition studies would be beneficial to a project teams to help develop potential new drugs. Furthermore, the technology should be one that can be used for several projects and be low in cost. We demonstrate that the Caliper LC3000 instrument provides this type of technology for several current projects within St. Louis Discovery and appears to be applicable for a number of future projects within St. Louis Discovery. Traditional kinase assays are end point assays which are difficult, time consuming and low throughput when used to produce progress curves. The LC3000 provides a format which can generate progress curves in a quick and timely manner as well as provide a higher throughput than the traditional assays.

The current capabilities of Caliper technology allow reactions to be performed in a 384-well plate at an average cost of approximately $0.10/well. The assay format described here is referred to as a "Off-Chip Mobility Shift Assay" and uses capillary electrophoresis on a microfludic chip to measure the conversion of fluorescently-labeled substrate to product. The measurement can either be done in a real time mode ("kinetic mode") or from a plate of quenched reactions. In the real time analysis, samples are sipped from a plate at specified intervals over a period of time through the capillaries and onto the chip where substrate and product are separated by electrophoresis. The same analysis is performed with the quenched reaction, however, in this case, each well is only sampled once.

Caliper-based kinase and protease assays have been used in St. Louis Discovery for medicinal chemistry and hit follow-up library support, subset library screening, detailed inhibitor characterization, and as orthogonal assays to compliment the HTS primary screen.

Off-chip Mobility Shift Assay

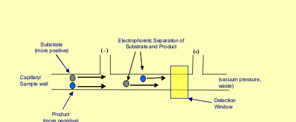

Figure 1. Diagram of Microfluidic "Sipper Chip". Samples are drawn into the interior channels of the Sipper Chip through the capillaries using vacuum pressure. Voltage is applied to the upstream and downstream electrode wells allowing for the separation of species with different electrophoretic mobilities. Fluorescently-labeled species are detected as they pass through the detection window of the Sipper Chip

Off-chip Mobility Shift Data Trace

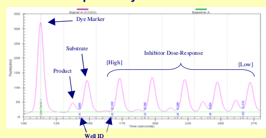

Figure 2. Analysis of data collected using the LabChip 3000. Caliper's HTS Well Analyzer software determines the heights of the corresponding product and substrate peaks. Data output files, including the product/sum ratio of peak heights, maybe reformatted using Excel macros.

Early Assay Development for a Lipid Kinase

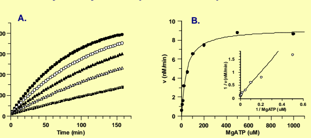

Figure 3. Assay Development for a lipid kinase. A) Lipid kinase activity in the presence of 1 µM FITC-lipid, 2.5 nM enzyme, and 500 µM (closed circles), 100 µM (open circles), 50 µM (closed triangles), 20 µM (open triangles), or 10 µM (closed squares) of MgATP. Wells were sampled by the capillaries every 3 minutes. Note: additional concentrations of MgATP were removed for clarity. B) Initial rates take from A., were fit to Equation 1 to yield K_M^{app} (MgATP).

Steady-State Characterization of a Ser/Thr Kinase

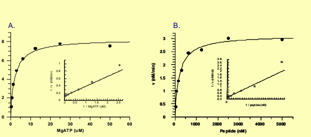

Figure 4. Characterization of steady-state kinetic parameters for a Ser/Thr kinase. A) Initial velocities were collected at several concentrations of MgATP and a fixed concentration of peptide (1 µM). The data were fit to Equation 1 to determine the apparent $K_{m\,(MgATP)}$. B) Initial velocities were collected at several concentrations of peptide and a fixed concentration of MgATP (2 µM). The data were fit to Equation 1 to determine the apparent $K_{m\,(peptide)}$.

Small Library Screen against a Lipid Kinase

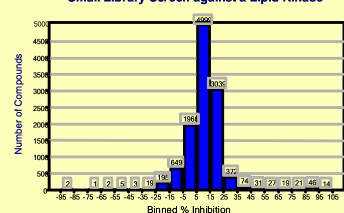

Figure 6. Compounds screened at a 10uM concentration (single-point) against a lipid kinase. The screening assay was performed at the Km level of MgATP and at less than Km levels of FITC-lipid.

Mechanism of Action Studies for the Inhibition of a Ser/Thr Kinase by PDXXX15

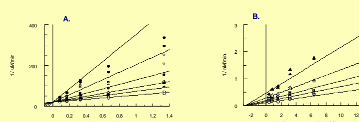

Figure 6. Characterization of the inhibition of a Ser/Thr Kinase by PDXXX15. A) PDXXX15 was varied at fixed concentrations of MgATP in the presence of 0.5 µM FITC-peptide. The data fit best to a competitive pattern of inhibition (Equation 2) with a K_{is} = 0.34 ± 0.032 µM. B) PDXXX15 was varied at fixed concentrations of FITC-peptide in the presence of 1.5 µM ATP. The data fit best to a noncompetitive pattern of inhibition (Equation 3) with a K_i = 0.45 µM.

Irreversible Inhibition of Ser/Thr Kinase by PHA-XXX883E

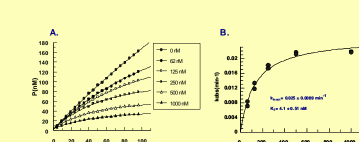

Figure 7. Kinetics of inhibition of a Ser/Thr kinase by PHA-xxx883E. A) Enzyme was diluted into MgATP (45 µM, 40XK_M), peptide and inhibitor at the indicated concentrations. The data was fit to progress curve describing irreversible inhibition (Equation 5). B) Second order plots of PHA-xxx883E. k_{obs} derived from figure 7A were fit to Equation 6

$T_{1/2}$ value determination of compounds against a Ser/Thr kinase

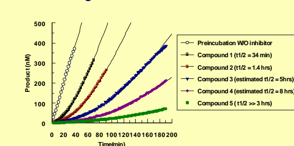

Figure 8. Inhibitor and enzyme were pre-incubated (50 nM enzyme and 50 nM inhibitor) for 1 hour at room temperature. The pre-incubation mixture was diluted into saturating amounts of MgATP (40X Km) to provide a final concentration of 0.1 nM enzyme and 0.1 nM inhibitor, respectively. The dissociation rates (koff) were determined using Equation 4.

Limitations to Consider

- Fluorescent substrate availability for an assay
- Only one phosphorylation site can be analyzed, current data package can not analyze two phospho sites
- Limited fluorescent substrate concentration range (50 nM-20 µM w/out instrument "tweaking")
- Charge and Mass of fluorescent substrate must be amenable to separation (generally, <20 AA and a charge of -5 to +5)
- Product conversion higher than desired for typical enzymology studies (>10%)
- Limited run time due to plate evaporation and buffer depletion (max run is typically 6 - 8 hours)

Summary

- High quality of data generated by the LC3000 due to the measurement of both substrate and product
- A single assay format may be used for assay development, small library screening, and detailed inhibitor characterization
- Assay development is done in a quick manner because of the LC3000 ability to generate real time readout
- The LC3000 supports screening of small libraries and allows for easy identification of any compounds interfering with fluorescence
- The LC3000 allows for steady-state inhibitor characterization as both ATP and peptide substrate may be varied
- Evaluation of inhibitor kinetics (i.e. on- and off- rates) may be moved higher in a project's testing scheme due to the ability to assay several compounds per run using the LC3000 in a "kinetic mode"
- Several Ser/Thr, tyrosine and lipid kinase projects are supported using the LC3000 technology at Pfizer-St. Louis