LabChip Mobility-Shift Assay: Phosphotidylinositol-3 Kinase PI3Kα

I. Introduction

Phosphotidylinositol-3 kinases (PI3Ks) generate phosphoinositide secondary messengers affecting a wide range of cellular responses, including the regulation of cell proliferation, cell survival, cell migration, membrane trafficking, and protein synthesis. Because the signaling pathways they trigger are among the most frequently activated pathways in cancer cells, PI3Ks are critical targets in many drug discovery programs. Caliper Life Sciences has developed a novel assay for Class IA PI3K activity utilizing a fluorescent-labeled phosphotidylinositol (4,5) diphosphate ($PI(4,5)P_2$) derivative. The addition of a phosphate group at the 3' position forms the phosphotidylinositol (3,4,5) triphosphate ($PI(3,4,5)P_3$) product, which can be electrophoretically separated from the substrate on the LabChip EZ Readers or LabChip 3000 instruments. This application note describes development and optimization of the LabChip Mobility-Shift assay using Invitrogen's Class IA PI3Kα (p110α/p85α).

II.Results

Separation of Substrate and Products

Figure 1 shows the structures of Caliper's flourescent-labeled $PI(4,5)P_2$ and $PI(3,4,5)P_3$. The molecules are labeled at the terminus of one of the two acyl groups with 5FAM, and provided as triethylamine ($C_6H_{15}NH^+$) salts. 5FAM-$PI(4,5)P_2$ is used as the substrate for PI3Kα assays, and 5FAM-$PI(3,4,5)P_3$ serves as a control for identification of the appropriate reaction product. As shown in Figure 2, the PIP_3 product is effectively separated from the PIP_2 substrate on the LabChip (red tracing). In addition, any formation of PIP_1, due to co-purifying phosphatase activity in the PI3K preparation, can easily be detected (blue tracing).

Enzyme Activity

PI3K preparations from several different vendors were tested in the Caliper assay; Figure 3 shows the results from 3 different PI3K preparations. Both kinase and phosphatase activity was observed with most preparations of PI3K. Reaction conditions were modified to maximize the kinase and minimize the phosphatase activity by varying buffer composition, NaCl concentration, detergent addition, DTT addition, and sodium cholate concentration (data not shown). Buffer composition was the only parameter which appreciably affected enzyme activity. As shown in Figure 3, enzymes showed significantly more kinase activity in MOPS pH 6.5 than in HEPES pH 7.5.

Attempts were made to inhibit phosphatase activities by adding orthovanadate, B-glycerophosphate, or PTEN inhibitors such as pbV(pic) (dipotassium bisperoxo (picolinato) oxovanadate V) to the reaction. In most cases, the inhibitors were ineffective. Experiments using active PTEN enzyme with 5-FAM-$PI(3,4,5)P_3$ demonstrated that bpV(pic) at 50 μM final concentration effectively inhibited the activity of PTEN, but not the phosphatase activity in the PI3K preparations (data not shown). Invitrogen's PI3Kα was chosen as the best enzyme preparation for further assay development, as the level of phosphatase activity was consistently low (Figure 2, red tracing, Figure 3, Inv PI3Kα). Using the optimized reaction buffer conditions described in Materials and Methods, 10-25% conversion of PIP_2 to PIP_3 was observed in 60-90 minutes with a final PI3Kα concentration of 10 nM.

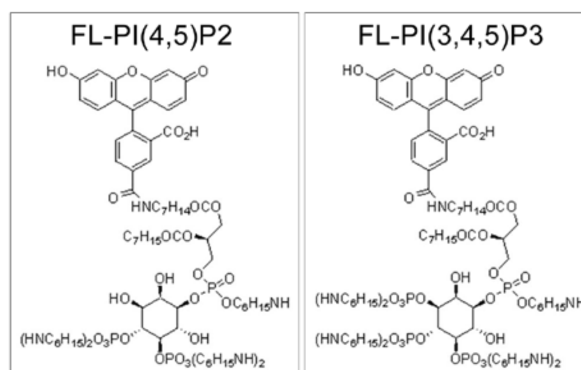

Figure 1. Caliper's fluorescent-labeled PIP_2 and PIP_3 substrates.

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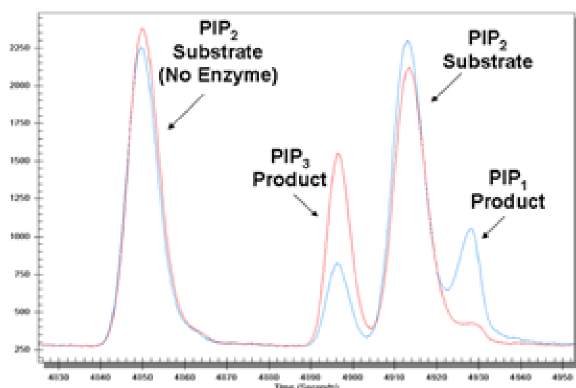

Figure 2. Sample data tracings showing the separation of PIP_3, PIP_2, and PIP_1. The red tracing is data from a reaction containing Invitrogen's PI3Kα. The blue tracing is data from a reaction containing pronounced phosphatase activity in a PI3Kα preparation supplied by a different vendor.

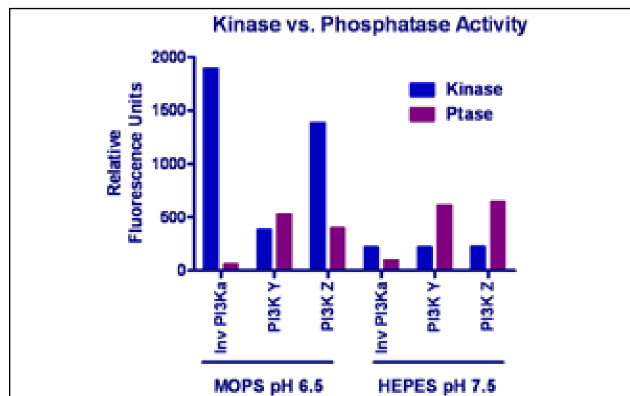

Figure 3. Relative Kinase and Phosphatase activities observed in three different PI3K preparations in buffer made with 50 mM MOPS pH 6.5 or 50 mM HEPES pH 7.5. All reactions contained 1.75 ng/µL enzyme, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM Na Cholate, 1 µM PIP_2, and 100 µM ATP. 'Inv PI3Kα' is Invitrogen's p110α/p85α.

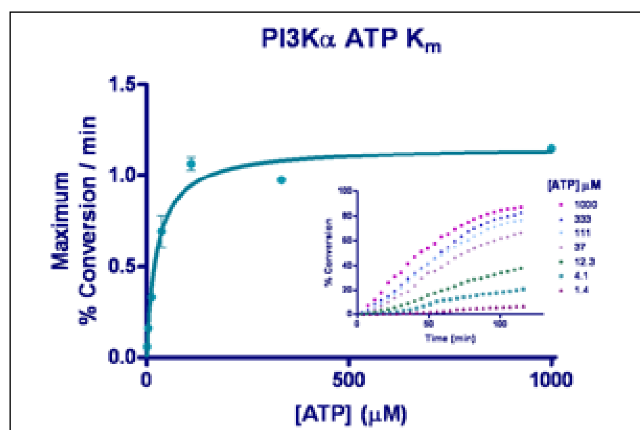

Figure 4. The ATP K_m^{app} for PI3Kα was determined to be 25.3 +/- 3.3 µM. The inset shows one example of progress curves from reactions containing varying concentrations of ATP.

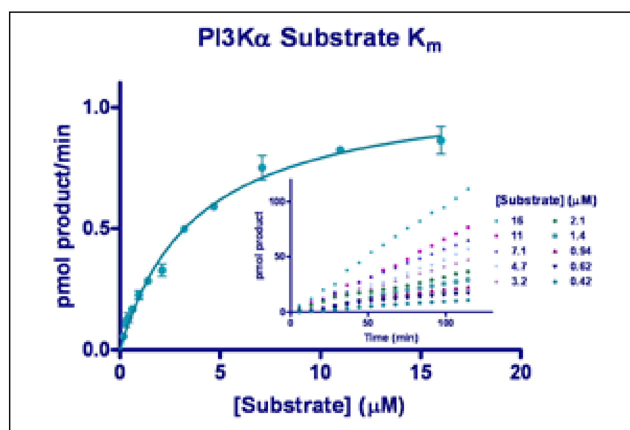

Figure 5. The Substrate K_m^{app} for PI3Kα was determined to be 3.7 +/- 0.4 µM. The inset shows one example of progress curves from reactions containing varying concentrations of PIP_2 substrate.

ATP K_m

Reactions containing 10 nM PI3Kα, 1 µM PIP_2 substrate and varying concentrations of ATP were assembled in a total volume of 70 µL and read on the EZReader II in kinetic mode to produce progress curves (Figure 4, inset). Using GraphPad Prism 5, maximum reaction rates for each well were calculated by linear regression analysis. Michaelis-Menten analysis of data from 4 independent experiments gave an ATP K_m^{app} value of 25.3 +/- 3.3 µM for the PI3Kα assay (Figure 4).

Substrate K_m

Reactions containing 10 nM PI3Kα, 25 µM ATP, and varying concentrations of PIP_2 were assembled in a total volume of 70 µL and read in kinetic mode. Percent conversion values from the EZReader II data analysis were converted to pmol product using the following equation:

$$\text{pmol product} = \frac{\text{\% conversion}}{100} \times \text{[substrate] (µM)} \times \text{rxn volume (µL)}$$

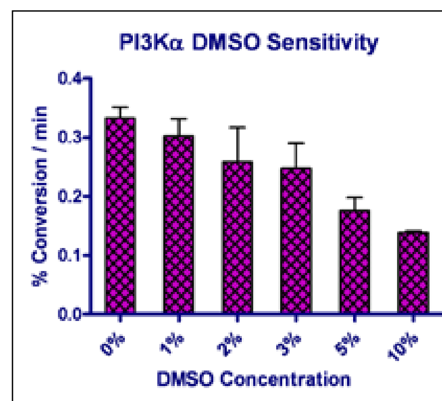

Figure 6. PI3Kα activity was sensitive to DMSO at concentrations above 2%.

The maximum reaction rates for each well were calculated by linear regression analysis of the progress curves (Figure 5, inset). Michaelis-Menten analysis of data from 4 independent experiments gave a PIP_2 Substrate K_m^{app} value of 3.7 +/-0.4 µM (Figure 5).

DMSO Sensitivity

Kinetic reactions containing 10 nM PI3Kα, 25 µM ATP, 1 µM PIP_2 substrate and varying concentrations of DMSO were assembled in a total volume of 70 µL. Linear regression analysis of the progress curves was performed to determine the reaction rates in % conversion per minute. Figure 6 shows the effect of DMSO concentration of reaction rates. Reactions containing > 2% DMSO progressed slower than those containing no DMSO. In order to retain >75% enzyme activity, it is important to keep the concentration of DMSO at or below 3%.

Reaction Progress Curves

Figure 7 shows average percent conversion observed in reactions containing varying concentrations of PI3Kα. All reactions were assembled with 25 µM ATP, 1 µM PIP_2, and 3% DMSO in a total volume of 70 µL and placed immediately into the EZReader II. In this experiment, reactions containing 15 nM enzyme demonstrated a lag time of approximately 30 minutes before reaching the maximum reaction rate (pink data points). This type of lag phase was frequently observed in PI3Kα reactions with enzymes from several vendors. Pre-activation of enzyme with 500 µM ATP did not eliminate the lag phase (data not shown). The duration of the lag phase was not reproducible from day to day, and may depend on enzyme handling and pipetting technique.

Due to the high variability of the lag phase, it is recommended to include enzyme activity controls in endpoint experiments. To ensure that endpoint reactions are stopped when 10-25% conversion has occurred, the % conversion in the enzyme activity controls can be checked by reading them on the EZReaders or LabChip 3000 after an incubation time of 45-60 min. The % conversion observed in these control reactions can be used to estimate the appropriate incubation time for the larger experiment. In addition, laboratory automation should be used wherever possible to avoid well-to-well differences in pipetting and mixing.

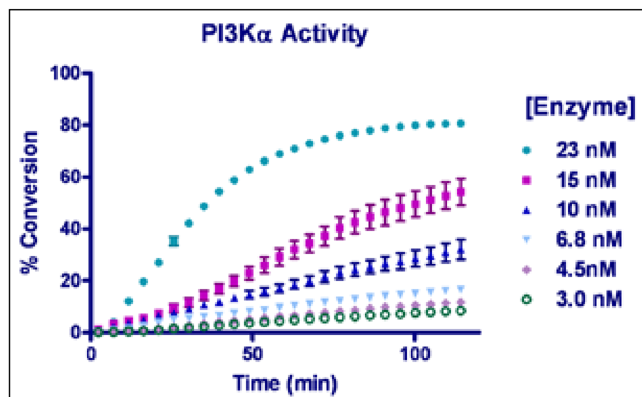

Figure 7. Progress curves for PI3Kα reactions run at 25 µM ATP and 1 µM PIP_2.

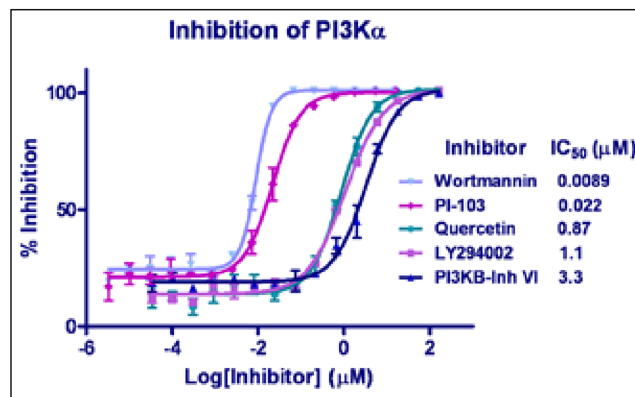

Figure 8. Effect of known inhibitors on PI3Kα activity

Inhibitor IC_{50} Determination

Endpoint reactions containing 10 nM enzyme, 25 µM ATP and 1 µM PIP_2 were run with varying concentrations of 5 known inhibitors of Class I PI3K inhibitors. After spotting 1 µL 31X inhibitor in 50% DMSO and adding 15 µL 2X enzyme, plates were pre-incubated for 15 minutes at room temperature (3% DMSO in pre-incubation mix). 15 µL 2X substrate and ATP was added, and plates were incubated for 75 minutes at room temperature. The reactions were stopped by the addition of 45 µL stop buffer and read in the EZReader II. Percent inhibition values were calculated in Caliper's EZReviewer Software, and plotted vs. the log of inhibitor concentration. GraphPad Prism was used to fit the data with dose-response curves and determine IC_{50} values (Figure 8). In all cases, the IC_{50} values determined with the LabChip assay were consistent with literature values.

Assay Consistency and Data Stability

An endpoint reaction experiment was set up to measure assay consistency at the lower end of the recommended % conversion range for the assay (10% conversion, Figure 9). One µL of 50% DMSO was spotted into each of 96 wells, then 15 µL of 2X Enzyme was added and the reactions were pre-incubated. After 15 minutes, 15 µL of 2X Substrate + ATP was added to 72 wells (No Inhibition) and 15 µL of 2X Substrate only was added to 24 wells (No ATP). The reactions were incubated for 1 hour at room temperature, terminated by the addition of 45 µL Stop Buffer, then read on the EZReader II. The Z' for this experiment was 0.6, a value lower than those usually observed with peptide-based assays on the LabChip platform. This is likely due to the heterodimeric nature of the enzyme and the variable lag time for the reaction. As discussed above, care should be taken to keep pipetting and mixing consistent between different wells, with use of automated pipetting wherever possible. In addition, use of enzyme activity controls to target 20-25% conversion will result in a more robust assay.

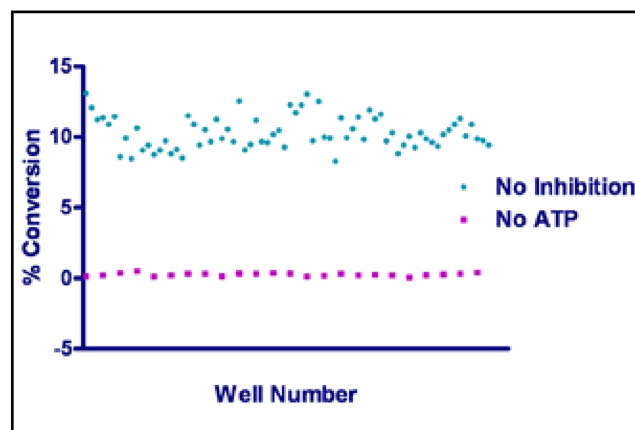

Figure 9. Data consistency for the PI3Kα assay. The Z' for the assay at 10% conversion was 0.6.

Figure 10 shows data obtained from 6 wells of a stopped endpoint reaction immediately after reaction termination and 12 hours later. Stopped reactions were kept at room temperature between reads on the EZReader II. The heights and shapes of the substrate and product peaks were consistent, indicating that both substrate and product are stable at room temperature.

III. Conclusions

Caliper's fluorescent-labeled $PI(4,5)P_2$ has been validated as a substrate for the LabChip mobility-shift assay for Invitrogen's PI3 kinase p110α/p85α (PI3Kα). The substrate is also suitable for assays with the other Class I PI3 kinases, PI3Kß and PI3Kδ (data not shown). While other technologies may be used for PI3K assays, the LabChip platform has the distinct advantage of simultaneously measuring phosphotidylinositol kinase and phosphatase activities in the same reaction well. In addition, PI3K reactions can be run either in endpoint mode, for screening and IC_{50} determination, or in kinetic mode to study enzyme kinetics and mechanisms of inhibition.

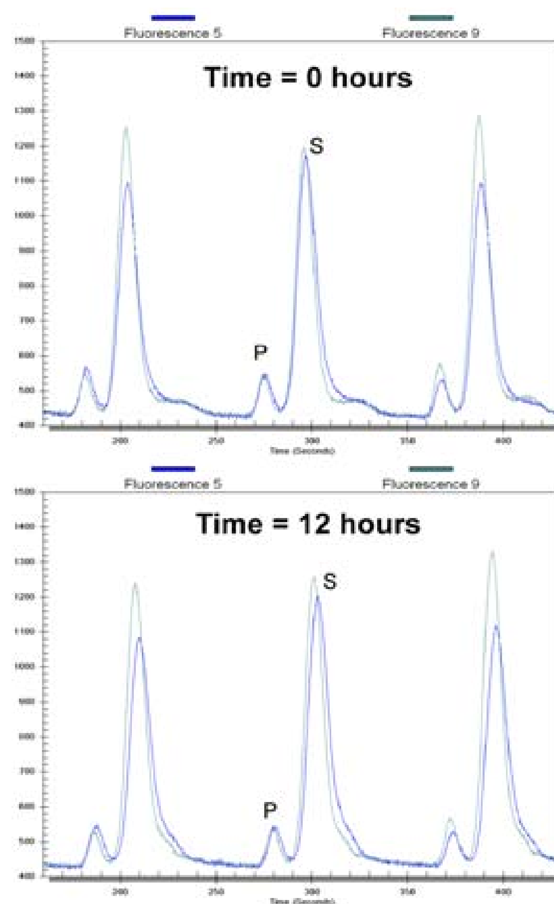

Figure 10. PI3Kα assay data tracings obtained immediately after stopping the reaction (Time = 0 hours) and 12 hours later show that the PIP_2 substrate (S) and PIP_3 product (P) are stable at room temperature.

IV. Materials and Methods

PI3K Reaction Buffer
50 mM MOPS, pH 6.5
10 mM $MgCl_2$
25 mM NaCl
0.5 mM NaCholate

ATP Concentration ($K_m$app)
25 µM

PIP2 Substrate Concentration
1.0 µM

Enzyme Concentration* (final in reaction)
10 nM

Stop Buffer
50 mM MOPS, pH 6.5
25 mM NaCl
0.5 mM NaCholate
30 mM Disodium EDTA

Endpiont Assay Setup
1 µL compound in 50% DMSO
15 µL 2X Enzyme
15 µL 2X Substrates (PIP_2 and ATP)
Incubate 90 min at Room Temp*
45 µL Stop Buffer

Separation Buffer
ProfilerPro Separation Buffer
+9 mM Disodium EDTA
+3X CR-8

Separation Conditions
Downstream Voltage: -2000 V
Upstream Voltage: -500 V
Pressure: -2.0 psi
Post-sample sip time: 60 seconds
Final delay: 120 seconds

Table 1. Optimized conditions for mobility-shift assays with Invitrogen's PI3Kα.

* Enzyme concentration and reaction time are enzyme-lot specific, and will need to be adjusted for each lot. Optimal concentrations should produce 20-30% conversion in 60-90 min.

ITEM	MANUFACTURER	CATALOG NUMBER
FL-PI(4,5)P2	Caliper Life Sciences	760456
FL-PI(3,4,5)P3	Caliper Life Sciences	760457
ProfilerPro Separation Buffer	Caliper Life Sciences	760367
Coating Reagent 8	Caliper Life Sciences	760278
PI3Kα (p110a/p85a), Lot # 368262C	Invitrogen	PV4788
Wortmannin	Calbiochem	681675
LY294002	Calbiochem	440204
PI3-KB Inhibitor VI, TGX-221	Calbiochem	528113
Quercetin, Dihydrate	Calbiochem	551600
PI-103	Calbiochem	528100
MOPS	Calbiochem	475898
Sodium Cholate	Sigma	C6445
Sodium Chloride, 5M solution	Ambion	9760G
Magnesium Chloride, 1M solution	Ambion	9530G
ATP	Sigma	A7699
EDTA, disodium salt, 0.5 M, pH 8.0	Sigma	E7889
DMSO	Sigma	D5879
18 MΩ water		

68 Elm Street
Hopkinton, MA 01748-1668

Tel: 1.508.435.9500
Email: cust.support@caliperLS.com

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