Prepare PI3K samples
Prepare kit reagents
Setup PI3K reactions at room temperature in OptiPlate
Prepare PI(3,4,5)P3 standards and add to the plate
Add PI(3,4,5)P3 Detector to OptiPlate
Add Red Fluorescent Probe to OptiPlate and incubate in the dark for 30-60 min.
Measure Fluorescence Polarization

<table>
<thead>
<tr>
<th>Catalog #</th>
<th>Description</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>K-1101L</td>
<td>PI(4,5)P2 Substrate</td>
<td>69 µg</td>
</tr>
<tr>
<td>K-1003s</td>
<td>PI(3,4,5)P3 Standard</td>
<td>0.88 µg</td>
</tr>
<tr>
<td>K-1103</td>
<td>PI(3,4,5)P3 Detector</td>
<td>1 pellet</td>
</tr>
<tr>
<td>K-1104</td>
<td>2.5 uM Red Fluorescent Probe</td>
<td>50 µL</td>
</tr>
<tr>
<td>---</td>
<td>OptiPlate-384 F</td>
<td>1 plate</td>
</tr>
<tr>
<td>K-KBZ</td>
<td>5X KBZ buffer</td>
<td>4 mL</td>
</tr>
<tr>
<td>K-ATP1</td>
<td>10 mM ATP</td>
<td>50 µL</td>
</tr>
<tr>
<td>K-DTTI</td>
<td>DTT</td>
<td>50 µmol</td>
</tr>
<tr>
<td>---</td>
<td>Acetate plate seal</td>
<td>1 seal</td>
</tr>
</tbody>
</table>

**Additional Materials Provided by User:**
- Source of PI3-Kinase. Enzyme may be immunoprecipitated or purified (PI3Kα Echelon cat# E-2000). Use of crude lysates is not recommended.
- Fluorescence plate reader equipped for Fluorescence Polarization using red fluorophores with appropriate filters (550 nm excitation/580 nm polarizing emission filters)

**Storage:** Upon receipt, store the kit at –20°C. Some components are light sensitive. Under proper storage conditions, this product is stable for at least 6 months from date of receipt. Opened and reconstituted solutions are less stable. All components and solutions should be protected from excessive light and heat.

Echelon Biosciences products are sold for research and development purposes only and are not to be incorporated into products for resale without written permission from Echelon Biosciences. This kit and all non-radioactive, competitive assays for determining phosphoinositide-3-kinase (PI3-K) activity are protected by Echelon Biosciences Inc. U.S. Patent 7,067,269. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. For inquiries email busdev@echelon-inc.com.
The production of PI(3,4,5)P3 from PI(4,5)P2 by PI3-Kinases (PI3-K) is important in multiple cell signaling pathways. Typically, experiments to measure PI3-K activity have involved phosphorylation of a phosphoinositide substrate using 32P, extraction of radioactive products, and separation using thin-layer chromatography. The fluorescence polarization PI3-K activity assay developed by Echelon Biosciences, Inc. allows the user to determine PI3-K activity using a homogenous mix and read format, eliminating the need for radioactivity, organic solvents, and thin layer chromatography. The assay was developed using a Fusion Alpha Universal Microplate reader equipped for Fluorescence Polarization. The sensitivity of the assay and the amount of substrate, detector, and fluorescent probe required for each assay point may vary depending on the specific fluorescence polarization detection system you are using. The provided OptiPlate-384 F assay plate works well with PI3K reactions. If you choose to use a different plate, please test PI3K reactions in the assay to make sure the well surface will not inhibit PI3-K reactions. Corning plates with NBS surface are not recommended. The concentrations of DTT, ATP, and PI(4,5)P2 substrate suggested are based on our experience using recombinant PI3Kα. The assay conditions used in your enzyme reaction can affect your enzyme activity and the activity of a potential inhibitor. You may want to titrate DTT, ATP, and/or PI(4,5)P2 substrate to determine the optimum conditions for your experiments.

Suggested controls include:
- Buffer Only: 5 μL of 2X Reaction Buffer, 5 μL of enzyme diluent, 15 μL of Detector Diluent. This control may be required as FP Blank for plate reading in FP mode.
- Probe Alone: 5 μL of 2X Reaction Buffer, 5 μL of enzyme diluent, 10 μL of Detector Diluent, and 5 μL of 50 nM Probe.
- No enzyme: 5 μL of 2X Reaction Buffer, 5 μL of enzyme diluent, 10 μL of PI(3,4,5)P3 Detector, and 5 μL of 50 nM Probe.

Reagents and Controls
- PI(3,4,5)P3 Standard (K-1003s) Add 22.4 μL of dH2O to the vial of PI(3,4,5)P3 for a 40 μM solution; vortex to fully reconstitute; spin down and leave vial at room temperature prior to use. Enough standard is provided for approximately 10 separate dilution series. Store frozen at –20°C after use for up to 6 months. Multiple freeze–thaw cycles do not affect stability.
- DTT (K-DTT1) Add 50 μL of dH2O to the vial of DTT for a 1 M solution and vortex to fully reconstitute; spin down and leave vial at room temperature prior to use. Enough standard is provided for approximately 10 separate dilution series. Store frozen at –20°C after use for up to 1 month.
- ATP (K-ATPI) One vial with 50 μL of 10 mM ATT. Store frozen at –20°C after use.
- PI(4,5)P2 Substrate (K-1101L) Add 100 μL of dH2O to the vial of PI(4,5)P2 for a 800 μM solution; vortex to fully reconstitute; spin down and leave vial at room temperature prior to use. There is substrate for up to 800 assay points using 100 pmol per assay point (Final concentration in reaction: 10 μM). Store frozen at –20°C after use for up to 6 months.
- 2X Reaction Buffer Dilute 5x KBZ buffer (K-KBZ) 2.5-fold in dH2O and supplement with 4 mM DTT, 20 μM PI(4,5)P2 substrate, and 50 μM ATP. Only prepare the amount needed. Make fresh before each use. Store remaining 5x KBZ at 4°C.

Technical Data Sheet Rev. 8. 07-12-18 - For research use only. Not intended or approved for diagnostic or therapeutic use. Page 2 of 4
For each 10 μL reaction:

- 5 μL 2X Reaction Buffer
- 5 μL of enzyme reactions, controls, or PI(3,4,5)P3 standards in each well.
- Note: we suggest running a Probe Alone and Buffer Only control with each experiment. See assay notes for more information.

2. Quenching and Detection of PI 3-Kinase Activity

Mix in the following order for a total of 25 μL per well in the 384-well plate provided with the kit:

a. 10 μL of enzyme reactions, controls, or PI(3,4,5)P3 standards in each well.
   - Note: we suggest running a Probe Alone and Buffer Only control with each experiment. See assay notes for more information.

b. 10 μL of 250 nM stock of PI(3,4,5)P3 Detector.
   - Note: Addition of this reagent quenches the kinase reaction. If you are running a time course of enzyme activity, the PI(3,4,5)P3 Detector solution can be added to quench at various time points and the Red Fluorescent Probe solution can be added after all reactions are complete.

c. 5 μL of 50 nM Red Fluorescent Probe working solution.

3. Incubation and Measurement

Tap plate to mix gently. Seal plate and protect from exposure to light. Incubate in a dark location for 30 - 60 minutes to equilibrate. Incubations may be as long as six hours with minimal effect on final measurements. Measure fluorescence polarization using an appropriate instrument and filter set compatible with TAMRA dyes (550 nm excitation/580 nm polarizing emission filters will give satisfactory results.). Values obtained for enzyme reactions can be compared to the standard curve to determine conversion of substrate to PI(3,4,5)P3.

### Table 1. PIP3 Standards

<table>
<thead>
<tr>
<th>Concentration of PIP3</th>
<th>PIP3/10μL</th>
<th>100 μM stock or previous dilution</th>
<th>1X Reaction Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0 μM</td>
<td>40 pmol</td>
<td>8 μL (40 μM PIP3, Stock Soln.)</td>
<td>72 μL</td>
</tr>
<tr>
<td>2.0 μM</td>
<td>20 pmol</td>
<td>40 μL (4 μM Solution)</td>
<td>40 μL</td>
</tr>
<tr>
<td>1.0 μM</td>
<td>10 pmol</td>
<td>40 μL (2 μM Solution)</td>
<td>40 μL</td>
</tr>
<tr>
<td>0.5 μM</td>
<td>5 pmol</td>
<td>40 μL (1 μM Solution)</td>
<td>40 μL</td>
</tr>
<tr>
<td>0.25 μM</td>
<td>2.5 pmol</td>
<td>40 μL (0.5 μM Solution)</td>
<td>40 μL</td>
</tr>
<tr>
<td>0.125 μM</td>
<td>1.25 pmol</td>
<td>40 μL (0.25 μM Solution)</td>
<td>40 μL</td>
</tr>
<tr>
<td>0.0625 μM</td>
<td>0.625 pmol</td>
<td>40 μL (0.125 μM Solution)</td>
<td>40 μL</td>
</tr>
<tr>
<td>0 μM</td>
<td>0 pmol</td>
<td>-</td>
<td>40 μL</td>
</tr>
</tbody>
</table>

In our hands, each assay point uses approximately 10 ng of 6xHis-tagged recombinant PI3-Kα (E-2000) per 100 pmol of PI(4,5)P2 substrate, with 1 hour incubation at 37°C.

### Protocol for Fluorescence Polarization Assay

Please read this entire section and the Assay Notes section before beginning the assay.

For 10 μL reaction add the following:

- 5 μL of enzyme reactions, controls, or PI(3,4,5)P3 standards in each well.
- 10 μL of enzyme reactions, controls, or PI(3,4,5)P3 standards in each well.
- 10 μL of 2X Reaction Buffer.
- 5 μL of 250 nM stock of PI(3,4,5)P3 Detector.

Incubate at 25 to 37°C for appropriate time period, depending on the activity of your enzyme. The exact amount of enzyme and conditions of incubation will vary with different enzyme preparations and will need to be optimized for each specific application.
Support Protocols

**Immunoprecipitation of PI3-Kinase**
1. Grow cells to 80% confluence in 10 cm dishes.
2. Induce quiescence by incubating overnight in serum-free medium containing 0.5% insulin-free BSA.
3. Remove medium, and stimulate cells with 100 nM insulin for 10 minutes at 37°C.
4. Remove solution and place cells on ice. Add 10 mL per dish of ice-cold Buffer A (137 mM NaCl, 20 mM Tris−HCl pH 7.4, 1 mM CaCl2, 1 mM MgCl2, and 0.1 mM sodium orthovanadate). Rinse three times with this solution.
5. Remove Buffer A and add 1 mL of ice cold Lysis Buffer (Buffer A plus 1% NP−40 and 1 mM PMSF). Keep plates on ice for 20 minutes.
6. Scrape cells from dish, transfer to 1.5 mL microfuge tubes. Centrifuge for 10 minutes at high speed to sediment insoluble material.
7. Transfer supernatent to new tubes, add 5 μL of anti-PI3 kinase antibody (Millipore, catalog # 06-195) to each tube. Incubate for one hour at 4°C with gentle rotation.
8. Add 60 mL of a 50% slurry of Protein A−agarose beads in PBS to each tube. Incubate with mixing for one hour at 4°C.
9. Collect immunoprecipitated enzyme by centrifuging 5 seconds, and wash with freshly prepared buffers as follows:
   - Three times with Buffer A plus 1% NP−40
   - Three times with 0.1 M Tris−HCl pH 7.4, 5 mM LiCl, and 0.1 mM sodium orthovanadate.
   - Twice with TNE (10 mM Tris−HCl pH 7.4, 150 mM NaCl, 5 mM EDTA) containing 0.1 mM sodium orthovanadate.
10. Remove last wash as completely as possible. Wash twice with 1X KBZ and proceed immediately with kinase reactions as described in the Basic Protocol.

**References**


**Related Products**

<table>
<thead>
<tr>
<th>Products</th>
<th>Catalog Number</th>
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<tbody>
<tr>
<td>Assays and Enzymes</td>
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<tr>
<td>PI3-Kinase alpha, active (PI3Ka)</td>
<td>E-2000</td>
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<td>PI3-Kinase Activity ELISA: Pico</td>
<td>K-1000s</td>
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<td>Class III PI3K Elisa Kit</td>
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<td>Antibodies</td>
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<td>Anti-PtdIns(3,4,5)P3 IgG</td>
<td>Z-P345b</td>
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<td>Biotinylated Anti-PtdIns(3,4,5)P3 IgM</td>
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