

5' PtdIns(3,4,5)P3 Phosphatase Fluorescence Polarization Activity Assay

K-1400 (384 wells)

Support: echelon@echelon-inc.com

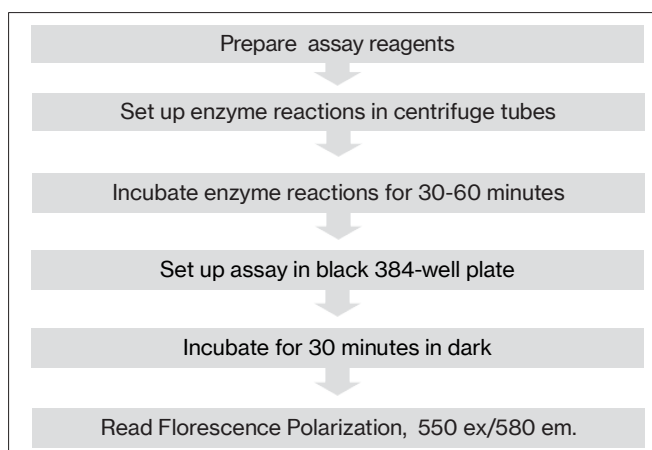
Description: The 5' PtdIns(3,4,5)P3 Phosphatase Activity Fluorescence Polarization Assay determines activity through quantification of the end product, PI(3,4)P2.

Storage: Some components are temperature and light sensitive. Store unopened kit at –20°C until use.

Materials Provided

Catalog #	Description	Quantity
K-1403L	PI(3,4,5)P3 (PIP3) Substrate, 10 µg	1 vial
K-1404L	PI(3,4)P2 Standard, 0.6 µg	1 vial
K-1405L	PI(3,4)P2 Detector, 10 µg	4 vials
K-1406	PI(3,4)P2 Probe, 200X	
K-PTAB	Phosphate Buffered Saline (PBS)	1 tablet
---	Black 384-well assay plate	1 plate
---	Microtiter Plate Seal	1 seal

Quick Protocol



Additional Materials Provided by User

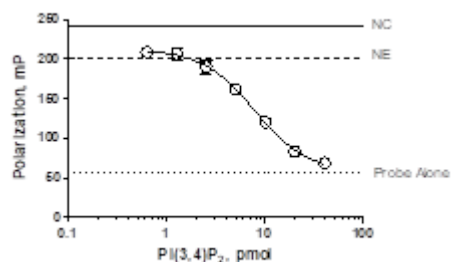
- Source of 5' PIP3 phosphatase (e.g. SHIP2)
- Reaction buffer (cat# K-S2RB for SHIP2)
- Fluorescence plate reader capable of Fluorescence Polarization detection with appropriate filters for red fluorophores (e.g. 550 nm excitation/580 nm polarizing emission)

Hazardous Properties and Cautions: The toxicological and pharmacological properties of this compound are not fully known. For further information see the MSDS on request. This product is manufactured and shipped only in small quantities, intended for research and development in a laboratory utilizing prudent procedures for handling chemicals of unknown toxicity, under the supervision of persons technically qualified to evaluate potential risks and authorized to enforce appropriate health and safety measures. As with all research chemicals, precautions should be taken to avoid unnecessary exposures or risks.

Warranty and Disclaimer: Echelon warrants the product conforms to the specifications stated herein. In the event of nonconformity, Echelon will replace products or refund purchase price, at its sole option, and Echelon shall not be responsible for any other loss or damage, whether known or foreseeable to Echelon. No other warranties apply, express or implied, including but not limited to warranty of fitness for any purpose or implied warranty of merchantability. Purchaser is solely responsible for all consequences of its use of the product and Echelon assumes no responsibility therefore, including success of purchaser's research and development, or health or safety of any uses of the product.

Assay Design

This FP assay is a competitive assay. After the phosphatase reactions are complete, reaction products are mixed with a PI(3,4)P₂ detector protein and the fluorescent PI(3,4)P₂ probe in the black 384-well assay plate. Polarization (mP) values decrease as probe binding to the PI(3,4)P₂ detector is displaced by PI(3,4)P₂ produced by enzymatic activity. The graph below shows typical results produced by increasing PI(3,4)P₂ amount on polarization values. Controls on graph: NC = No Competitor control; NE = No Enzyme control; PA = Probe Alone control. Please see Quick Assay Reference Guide on next page for details.



Assay Notes

- The assay was developed using a Perkin Elmer Fusion Alpha 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 instrument you are using. Please contact your plate-reader manufacturer or Echelon for assistance in modifying the protocol for your use.
- Bring assay plate, Substrate (K-1403L), and Standard (K-1404L) to room temperature prior to use. Keep remaining reagents on ice until use.

Reagent Preparation

- PBS buffer:** Dissolve PBS tablet in 200 mL of ddH₂O for PBS buffer. PBS buffer is stable at room temperature.
- PIP₃ Substrate:** Reconstitute the diC8 PIP₃ (K-1403L, 10 µg, 10.2 nmol) in 255 µL ddH₂O for a stock solution of 40 µM. The substrate is enough for 400 assay points using 20 pmol per point (concentration in reaction: 2 µM). Store stock solution frozen at -20°C for up to 3 months. Multiple freeze-thaw cycles do not affect stability. On day of assay, dilute 10-fold in appropriate reaction buffer for a 4 µM working solution.
- Standard Buffer:** Prepare a standard buffer containing 2 µM of PIP₃ substrate by diluting the 40 µM PIP₃

stock solution 20-fold in the appropriate reaction buffer.

- PI(3,4)P₂ Standard:** Reconstitute the vial of diC8 PI(3,4)P₂ (K-1404L, 0.6 µg, 0.7 nmol) in 350 µL ddH₂O for a stock solution of 2 µM. There is enough PI(3,4)P₂ for 4 triplicate dilution series. Store stock solution frozen at -20°C for up to 3 months. Multiple freeze-thaw cycles do not affect stability. On day of assay, make five 2-fold serial dilutions from the 2 µM stock solution in the prepared Standard Buffer above. See table below for an example of PI(3,4)P₂ standard dilution series.

Concentration of PI(3,4)P ₂ Standard	PI(3,4)P ₂ Standard / 10 µL
2.0 µM (Stock)	20 pmol
1.0 µM	10 pmol
0.5 µM	5 pmol
0.25 µM	2.5 pmol
0.125 µM	1.25 pmol
0.0625 µM	0.625 pmol
0 µM (No Competitor Control)	0 pmol

- PI(3,4)P₂ Detector:** Four vials of PI(3,4)P₂ detector are provided for use in up to 4 separate tests. Each vial of K-1405L contains 10 µg lyophilized PI(3,4)P₂ detector. This is enough detector for up to 96 assay points. Store at -20°C or at -80°C. Prior to use, reconstitute each vial of PI(3,4)P₂ detector in 1.0 mL PBS for a 10 µg/mL working solution. Keep the solution on ice. Reconstitute only the vial(s) as required for use that day. Each data point needs 10 µL of detector working solution. Invert the vial 5-10 times to mix gently and spin down right before use. If more than 1 vial is used, pool all the working solution together, mix gently, and spin down before use. Do not vortex the detector.
- Probe (200X):** One vial contains 11 µL of 200x concentrated BODIPY® TMR-labeled PI(3,4)P₂ probe. **IMPORTANT:** Minimize exposure of probe to light. Dilute probe (K-1406) 200-fold in PBS for a 50 nM working solution immediately prior to use. Each data point needs 5 µL of probe working solution.

5' Phosphatase Enzyme Reaction

(An Example Using Recombinant His-tagged SHIP2)

Enzyme concentration, reaction buffer, reaction temperature and reaction time for enzyme activity will depend on the characteristics and source of the enzyme used in each specific application. The following protocol has been used at Echelon to detect the activity of a recombinant His-tagged SHIP2 (cat# E-1000) and is provided as a guideline only. If you have an

established protocol for detecting phosphatase activity, that may be used. This assay will not tolerate the presence of BSA.

Set up reactions in micro centrifuge tubes (10 μ L reaction per assay point). A 40 μ L reaction should be enough for triplicate assay points.

Make a 4 μ M (2x concentrated) working solution of PIP3 substrate by diluting the 40 μ M stock solution 10-fold in SHIP2 reaction buffer (cat# K-S2RB). 5 μ L of the 4 μ M working solution will be used per assay point, to give 20 pmol per point.

For each 40 μ L reaction add:

1. 20 μ L of 4 μ M (2x conc.) PIP3 substrate working solution (2 μ M final concentration)
2. 20 μ L of 2x concentrated (0.5 to 1 ng/ μ L) SHIP2 enzyme diluted in SHIP2 reaction buffer

Close cap tightly and incubate at 37°C for 30 to 60 min. Stop reaction by heating the tube at 95°C for 3 min. Cool down to room temperature and centrifuge for a full minute to collect the reaction at the bottom of the tube.

Depending on the activity of your enzyme, the enzyme concentration and condition of reaction will need to be optimized. If enzyme activity is low, increase enzyme concentration or prolong reaction time. If enzyme activity is too high (~100% substrate conversion), reduce enzyme concentration, shorten reaction time, or lower reaction temperature to 30°C or 25°C.

Fluorescence Polarization Assay

1. Assay Setup (It's recommended to setup duplicate or triplicate assay points for each reaction, standard or control):
Add to each well of the black 384-well plate in the following order (refer to Quick Assay Reference Guide below):
 - a. Appropriate volume of reaction buffer and PBS to corresponding control wells.
 - b. 5 μ L of 4 μ M (2x conc.) PIP3 Substrate to No Enzyme control.
 - c. 10 μ L of Standard dilution series to corresponding standard wells.

Quick Assay Reference Guide (25 μ L total volume per assay point)

Control	Reaction Buffer	4 μ M PIP ₃ Substrate (2x conc.)	Enzyme (2x conc.)	PI(3,4)P ₂ Std	PBS	PI(3,4)P ₂ Detector	Probe
Reaction	---	5 μ L	5 μ L	---	---	10 μ L	5 μ L
Standard	---	---	---	10 μ L	---	10 μ L	5 μ L
No Enzyme (NE)	5 μ L	5 μ L	---	---	---	10 μ L	5 μ L
No Competitor (NC)	10 μ L	---	---	---	---	10 μ L	5 μ L
Enzyme Background	5 μ L	---	5 μ L	---	---	10 μ L	5 μ L
Probe Alone (PA)	10 μ L	---	---	---	10 μ L	---	5 μ L
Buffer Blank (Blank)	10 μ L	---	---	---	15 μ L	---	---

- d. 5 μ L of enzyme (2x concentrated) in reaction buffer to Enzyme Background control wells.
- e. 10 μ L stopped enzyme reaction mixture to corresponding reaction wells
- f. 10 μ L of PI(3,4)P₂ detector working solution to each well except for Probe Alone and Blank controls.
- g. 5 μ L of 50 nM Probe working solution to each well except for Blank control.

2. Incubation and Measurement

Tap plate a few times gently to mix. Seal plate and protect from light. Incubate in a dark location for 30 minutes to one hour to equilibrate. Incubation may be as long as six hours with minimal effect on final measurements.

Measure fluorescence polarization using an appropriate plate reader and filter set compatible with BODIPY® TMR dye. (550 nm excitation/580 nm polarizing emission filters will give satisfactory results.). Assign Buffer Blank wells as FP Blank before reading the plate.

Plot a PI(3,4)P₂ standard curve using non-linear regression curve fit (e.g. Sigmoidal dose-response (variable slope), see graph on second page for example). Polarization values obtained from enzyme reactions can then be used to interpolate PI(3,4)P₂ values in log pmol from the standard curve. PIP3 substrate conversion can thus be quantified.