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Technical Data Sheet

For research use only
Not intended or approved for diagnostic or therapeutic use.

5' PtdIns(3,4,5)P_3 Phosphatase Fluorescence Polarization Activity Assay

Catalog No.: K-1400

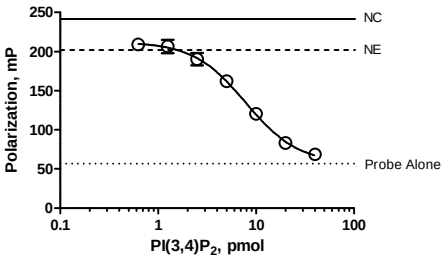
Storage:
Some components are temperature and light sensitive. Store unopened kit at –20°C until use.

Kit Contents:
1. K-1403L, PI(3,4,5)P_3 (PIP_3) Substrate, 10 µg
2. K-1404L, PI(3,4)P_2 Standard, 0.6 µg
3. K-1405L, PI(3,4)P_2 Detector, 4 vials of 10 µg/vial
4. K-1406, Probe (200X), 10 µM stock
5. K-PTAB, Phosphate Buffered Saline (PBS) tablet
6. Black 384-well assay plate
7. Acetate plate sealer

Researcher provides:
1. Source of 5' PIP_3 phosphatase (e.g. SHIP2)
2. Reaction buffer (cat# K-S2RB for SHIP2)
3. Fluorescence plate reader capable of Fluorescence Polarization detection with appropriate filters for red fluorophores (e.g. 550 nm excitation/580 nm polarizing emission)

Product description:

This FP assay is a competitive assay. After the phosphatase reactions are complete, reaction products are mixed with a PI(3,4)P_2 detector protein and the fluorescent PI(3,4)P_2 probe in the black 384-well assay plate. Polarization (mP) values decrease as probe binding to the PI(3,4)P_2 detector is displaced by PI(3,4)P_2 produced by enzymatic activity. The graph at right shows typical results produced by increasing PI(3,4)P_2 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.



Reagent Preparation:

PBS buffer: Dissolve PBS tablet in 200 mL of ddH_2O for PBS buffer. PBS buffer is stable at room temperature.

NOTE: Bring assay plate to room temperature prior to use.

PIP_3 Substrate: Reconstitute the vial of diC_8 PIP_3 (K-1403L, 10 µg, 10.2 nmol) in 255 µL ddH_2O 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 PIP_3 substrate by diluting the 40 µM PIP_3 stock solution 20-fold in the appropriate reaction buffer.

PI(3,4)P_2 Standard: Reconstitute the vial of diC_8 PI(3,4)P_2 (K-1404L, 0.6 µg, 0.7 nmol) in 350 µL ddH_2O for a stock solution of 2 µM. There is enough PI(3,4)P_2 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 at right for an example of PI(3,4)P_2 standard dilution series.

Concentration of PI(3,4)P_2 Standard	PI(3,4)P_2 Std per 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

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PI(3,4)P_2 Detector: Four vials of PI(3,4)P_2 detector are provided for use in up to 4 separate tests. Each vial of K-1405L contains 10 µg lyophilized PI(3,4)P_2 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)P_2 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)P_2 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 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 µL reaction per assay point). A 40 µL reaction should be enough for triplicate assay points.

Make a 4 µM (2x concentrated) working solution of PIP_3 substrate by diluting the 40 µM stock solution 10-fold in SHIP2 reaction buffer (cat# K-S2RB). 5 µL of the 4 µM working solution will be used per assay point, to give 20 pmol per point.

For each 40 µL reaction add:

1. 20 µL of 4 µM (2x conc.) PIP_3 substrate working solution (2 µM final concentration)
2. 20 µL of 2x concentrated (0.5 to 1 ng/µL) 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:

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

ID	Reaction Buffer	4 µM PIP_3 Substrate (2x conc.)	Enzyme (2x conc.)	PI(3,4)P_2 Std	PBS	PI(3,4)P_2 Detector	Probe
Reaction	---	5 µL	5 µL	---	---	10 µL	5 µL
Standard	---	---	---	10 µL	---	10 µL	5 µL
No Enzyme (NE)	5 µL	5 µL	---	---	---	10 µL	5 µL
No Competitor (NC)	10 µL	---	---	---	---	10 µL	5 µL
Enzyme Background	5 µL	---	5 µL	---	---	10 µL	5 µL
Probe Alone (PA)	10 µL	---	---	---	10 µL	---	5 µL
Buffer Blank (Blank)	10 µL	---	---	---	15 µL	---	---

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 the Quick Assay Reference Guide above):

a. Appropriate volume of reaction buffer and PBS to corresponding control wells.
b. 5 µL of 4 µM (2x conc.) PIP_3 Substrate to No Enzyme control.
c. 10 µL of Standard dilution series to corresponding standard wells.
d. 5 µL of enzyme (2x concentrated) in reaction buffer to Enzyme Background control wells.
e. 10 µL stopped enzyme reaction mixture to corresponding reaction wells
f. 10 µL of PI(3,4)P_2 detector working solution to each well except for Probe Alone and Blank controls.
g. 5 µL of 50 nM Probe working solution to each well except for Blank control.

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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,)P_2 standard curve using non-linear regression curve fit (e.g. Sigmoidal dose-response (variable slope), see graph on first page for example). Polarization values obtained from enzyme reactions can then be used to interpolate PI(3,4,)P_2 values in log pmol from the standard curve. PIP_3 substrate conversion can thus be quantified.

NOTE: 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.

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