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Technical Data Sheet

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

Product Name:

5' PtdIns(3,4,5)P₃ 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:

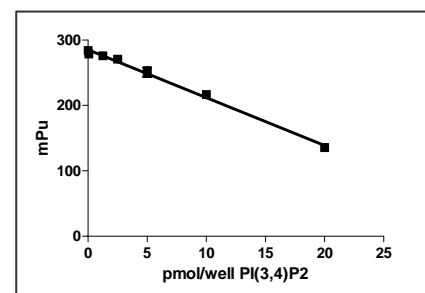
1. 1 vial PIP₃ Substrate 10X; 256 µl of 40 µM stock
2. 1 vial PI(3,4)P₂ Standard; 350 µl of 2 µM stock
3. 1 vial PI(3,4)P₂ Detector; 400 reactions/tube
4. 1 vial Probe (200X); 11 µl of 10 µM stock
5. Phosphate buffered saline (PBS) tablet
6. 1 black 384 well plate

Researcher provides:

1. Source of 5' phosphatase (cat#E-1000)
2. Buffers for reactions
3. Acetate plate sealers or equivalent
4. Fluorescence plate reader capable of Fluorescence Polarization detection with appropriate filters for red fluorophores (for example, 550 nm excitation/580 nm polarizing emission filters).

Background:

This 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. 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 shows typical results produced by increasing PI(3,4)P₂ concentrations on polarization values.



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Reagent Preparation:

PIP₃ Substrate 10X: One tube containing 256 µl of a 40 µM solution of diC₈ PI(3,4,5)P₃, enough substrate for 425 assay points using 20 pmols per assay point (concentration in reaction: 2 µM). Store frozen at -20 °C. Multiple freeze-thaw cycles do not affect stability. Dilute 1:10 in nanopure H₂O for a 4 µM working stock.

PI(3,4)P₂ Standard: One tube containing 350 µl of 2 µM solution of diC₈ PI(3,4)P₂, enough for approximately 4 separate dilution series. Store frozen at -20 °C. Multiple freeze-thaw cycles do not affect stability.

PI(3,4)P₂ Detector: One tube containing enough PI(3,4)P₂ detector for 400 assay points. Store at -20 °C.

This reagent is not stable at 4 °C. Dilute stock solution to 0.25 µM using the following equation to determine the volume of PBS to add to what volume of PI(3,4)P₂ detector required for use that day:

$\frac{1.25 \mu\text{M}}{\text{Final Conc.}} \times \frac{\text{Final Volume}}{\text{(for day's use)}} / \frac{\mu\text{M}}{\text{Detector Conc. (on tube)}} = \frac{\mu\text{l}}{\text{Volume Detector}}$
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Mix only enough reagent as required for use that day. Each data point uses 10 µl of 1.25 µM PI(3,4)P₂ Detector. For an entire plate, you will want 3,900-4,000 µl Final Volume. **Keep this reagent on ice at all times.**

Probe (200X): One tube containing 11 µl of 200 times concentrated BODIPY[®] TMR-labeled probe. Store frozen at -20 °C. **IMPORTANT: Minimize exposure of this reagent to light.** Dilute stock solution of probe 1:200 in PBS for a 50 nM working solution immediately prior to assay.

PBS buffer: Dissolve PBS tablet in 200 ml of purified water. Final concentration: 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl. Buffer may be stored at room temperature for 1 month.

NOTE: Bring plate to room temperature prior to assay.

5' Phosphatase reaction:

Amounts and exact conditions 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 and is given as a guideline

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only. If you have an established protocol for detecting phosphatase activity, that may be used. However, the assay will not tolerate the presence of BSA in buffers.

Set up reactions in 10 μ l volume per assay point.

Make a 4 μ M working solution of PIP₃ substrate by diluting the 40 μ M stock solution 1:10 in water. 5 μ l of the 4 μ M solution will be used per assay point, to give 20 pmols substrate per assay point. Substrate concentration in the reaction is 2 μ M.

Concentration of PI34P ₂ Standard	pmol/10 μ l PI34P ₂ standard
2.0 μ M (Stock)	20
1.0 μ M	10
0.5 μ M	5
0.25 μ M	2.5
0.125 μ M	1.25
0.0625 μ M	0.625
0 μ M (No Competitor Control)	0

For each 10 μ l reaction add:

1. 5 μ l of enzyme diluted in PBS with 10mM MgCl₂ (final concentration of MgCl₂ in reaction is 5mM)
2. 5 μ l of 4 μ M PIP₃ substrate (20 pmols, 2 μ M final concentration)

Incubate at room temperature 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. Using Echelon's recombinant His-tagged SHIP2 (1 pmol), 20 pmols of PIP₃ substrate is quantitatively converted to PI(3,4)P₂ within 30 minutes at room temperature (~25°C).

NOTE: We suggest running several assay points for each enzyme reaction. Enzyme reactions can be scaled up and divided into several assay points if desired. For example, for 4 assay points, set up a 40 μ l enzyme reaction containing 80 pmols (in 20 μ l) of PIP₃ substrate and 20 μ l of enzyme. For detection, add 10 μ l of the reaction mix per assay point for 4 replicate assay points.

Fluorescence Polarization Assay:

1. Assay Setup-Standards and Controls:

Along with the enzyme reaction mixtures, set up a standard curve of diC₈ PI(3,4)P₂ to allow determination of the amount of PI(3,4)P₂ produced. Make five 2-fold serial dilutions of the 2 μ M stock solution of PI(3,4)P₂ Standard in PBS to make the points outlined in the following table. Use 10 μ l of each dilution to set up a standard curve containing 0 (as a no competitor control) 0.625, 1.25, 2.5, 5, 10, and 20pmols PI(3,4)P₂. Also set up a probe alone control and a no enzyme control (as outlined below). It is suggested that standards and controls be run in duplicate or triplicate.

2. Detection of SHIP₂ activity:

Add to each well of the black 384 well plate in the following order:

1. 10 μ l of 1.25 μ M PI(3,4)P₂ detector per well (except for probe alone control, use PBS)
2. 10 μ l of Standard dilution series **or**
10 μ l enzyme reaction mixtures **or**
5 μ l PBS/ 5 μ l of 4 μ M PIP₃ Substrate (no enzyme control) **or**
10 μ l PBS (probe alone control)
in each well
3. 5 μ l of 50 nM Probe in each well (0.25 pmol per assay point)

Note: Total volume per well =25 μ l final volume

3. Incubation and Measurement:

Seal plate and protect from light. Incubate in a dark location for 15 minutes to one hour 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 BODIPY[®] TMR dye. (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 quantify conversion of substrate to PI(3,4,)P₂.

NOTE: The assay was developed using a Perkin Elmer Fusion 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.