Echelon Biosciences Inc.

INPP4 Activity Kit

K-5200 (96 tests)

Support: echelon@echelon-inc.com

Description: 96-well ELISA Assay for Detection of PI(3)P

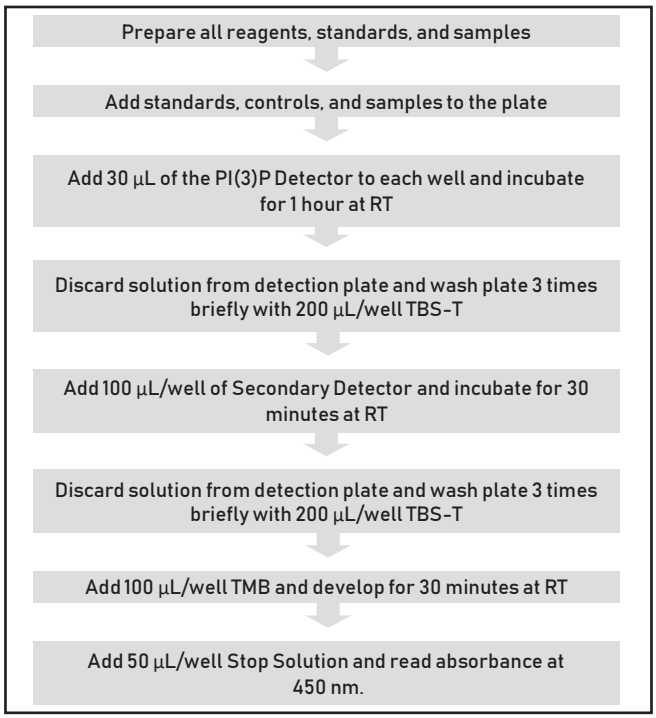
Materials Provided

Catalog #	Description	Amount
K-3001	Detection Plate, 12 x 8-strip well plate	1 Plate
P-3408	PI(3,4)P2 Substrate, diC8	100 µg
K-3003	PI(3)P Standard, diC8	3 µg
K-3004	5x PI(3)P Detection Buffer (5x DB)	4 mL
E-8000	INPP4A Enzyme Positive Control	2.5 µg
K-5202	5x INPP4 Reaction Buffer	1 mL
K-TBST	10X TBS-T Buffer	20 mL
K-3305	PI(3)P Detector	4 x 10 µg/vial
K-SEC2	Secondary Detector	300 µL
K-TMB1	TMB Solution	12 mL
K-STOPt	1 N H_2SO_4 Stop Solution	10 mL
---	Clear acetate sheet, plate seal	3 pieces

Additional Materials Provided by User

- INPP4 samples for testing
- Absorbance plate reader capable of reading at 450 nm

Quick Protocol



Storage

Store kit part 1 of 2 at 4°C. Store kit part 2 of 2 at -80°C.

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Ph: 801-588-0455
Fax: 801-588-0497

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Background

Inositol polyphosphate phosphatase 4 (INPP4) is also known as inositol polyphosphate 4-phosphatase and inositol-3,4-bisphosphate 4-phosphatase and has two isoforms known as INPP4A and INPP4B or INPP4 type I and type II. Both isoforms are magnesium-independent phosphatases involved in the phosphatidylinositol 3-kinase (PI3K) pathway and hydrolyze the 4 phosphate from the lipid second messenger phosphatidylinositol-3,4-bisphosphate (PI(3,4)P2) to form phosphatidylinositol-3-phosphate (PI(3)P). INPP4A suppresses glutamate excitotoxicity in the central nervous system and is responsible for the Weeble mouse phenotype[1, 2, 3]. INPP4B has been shown to regulate PI3K/AKt signaling, is lost in human basal-like breast cancers and may be a tumor suppressor in epithelial carcinomas[4].

Assay Design

Echelon's INPP4 Activity Assay is a competitive ELISA that tests the activity of INPP4A and INPP4B. The signal is inversely proportional to the amount of PI(3)P produced. After the INPP4 reactions are complete, reaction products are added to the PI(3)P-coated microplate for competitive binding to a PI(3)P detector protein. The amount of PI(3)P detector protein bound to the plate is determined through colorimetric detection. The ELISA is sensitive and specific; as low as 6 pmol PI(3)P can be detected in a 100 µL detection mixture (60 nM). This value corresponds to a sensitivity of 24 pmol in the original 20 µL reaction sample (1.2 µM). The assay displays excellent assay robustness (Z' value ~ 0.8).

Assay Notes

1. The PI(3)P standard curve is a PI(3,4)P2 to PI(3)P conversion curve, suitable for INPP4 reactions that produce 6 pmol to 200 pmol PI(3)P from 200 pmol initial PI(3,4)P2 substrate per assay point. Reaction conditions can be adjusted to produce 10 pmol to 100 pmol PI(3)P - the accurate PI(3)P measuring range.
   a. The following measures can be taken to increase PI(3)P production:
      - Increase the amount of INPP4 sample per reaction.
      - Perform INPP4 reactions at 37°C.
      - Increase reaction time.
   b. The following measures can be taken to decrease PI(3)P production:
      - Decrease the amount of INPP4 sample per reaction.
      - Lower INPP4 reaction temperature to 30°C or to room temperature.
      - Shorten reaction time.
2. This INPP4 activity assay can be modified to perform INPP4A inhibitor assay using 4-5 nM INPP4A enzyme (E-8000) with 40 µM PI(3,4)P2 substrate in each 20 µL reaction for 1 hour at 37°C. If it is desired to lower the PI(3,4)P2 substrate concentration below 40 µM in an inhibitor study, PI(3,4)P2 can be excluded from the PI(3)P standards since the PI(3,4)P2 competition would be negligible. In that case, prepare PI(3)P standards with 0.1x RB instead of SCB. Re-titer the INPP4A concentration and optimize reaction conditions may be desired.
3. Placing PI(3,4)P2 and PI(3)P at room temperature instead of on ice before and after reconstitution significantly increases INPP4A activity and keeps PI(3)P competition sensitivity consistent due to enhanced lipids solubility in solution.

Assay Protocol

Please read this entire section and Assay Notes before beginning.

Phosphatase Reaction

1. Isolate or prepare INPP4 enzyme samples using standard protocols. A vial of 2.5 µg of active INPP4A enzyme (cat# E-8000) is provided in the kit as enzyme positive control. Place control INPP4A enzyme (E-8000), INPP4 enzyme samples, PI(3)P Detector (K-3305), and Secondary Detector (K-SEC2) on ice. Bring all other reagents and Detection Plate (K-3001) to room temperature prior to use.
2. Prepare 200 µM of PI(3,4)P2 substrate stock by adding 583 µL of ddH2O to the vial of PI(3,4)P2 (P-3408). Vortex the vial shortly to fully reconstitute the lipid and place at room temperature until use. One vial of PI(3,4)P2 substrate contains enough for 100 reactions at 800 pmol of diC8 PI(3,4)P2 per reaction (enough for triplicate assay points). After use, store PI(3,4)P2 stock solution at -20°C for up to 3 months.
3. Pulse spin lyophilized control INPP4A enzyme (E-8000). Add 233 µL ddH2O and gently pipette up and down 5-10 times to make a 100 nM stock. Do not vortex. Place on ice until use. After use, aliquot, flash freeze, and store control enzyme at -80°C. Repeated freeze/thaw cycles may affect enzymatic activity.
4. Make a No Enzyme control mixture by labeling an eppendorf tube "NE" and adding:
   - 4 µL 5x Reaction Buffer (K-5202)
   - 4 µL of 200 µM PI(3,4)P2 (Step 2)
   - 12 µL deionized water
5. Make an INPP4A positive control mixture by labeling an eppendorf tube "C+" and adding:
   - 4 µL 5x Reaction Buffer (K-5202)
   - 4 µL of 200 µM PI(3,4)P2 (Step 2)
   - 8 µL deionized water
   - 4 µL resuspended 100 nM INPP4A (Step 3)
6. For each INPP4 sample to be tested, label an eppendorf tube appropriately and add:
   - 4 µL 5x Reaction Buffer (K-5202)
   - 4 µL of 200 µM PI(3,4)P2 (Step 2)
   - Enough deionized water to bring the reaction volume to 20 µL total
   - INPP4 sample

   It is recommended that each reaction be repeated in the absence of PI(3,4)P2 as control for PI(3)P in the sample preparation.
7. Let the phosphatase reaction proceed for 10 - 60 min at 25°C, 30°C or 37°C.
8. Quench by heating reactions at 95°C for ten minutes followed by cooling on ice for one minute then spinning at maximum speed in a microcentrifuge for one minute. Place reaction samples at room temperature until use in ELISA assay.

PI(3)P Detection

1. Add 180 µL ddH2O to each INPP4 reaction and mix well. This will provide enough sample for triplicate assay points in the following detection step.
   It's important to dilute INPP4 reaction 10-fold in water. Too high a strength of the reaction buffer in detection mixture will interfere with the PI(3)P detection.
2. Add 30 µL of 5x Reaction Buffer (K-5202) to 1.47 mL ddH2O for 1.5 mL of 0.1x Reaction Buffer (0.1x RB).
3. Add 1024 µL of 0.1x RB to the vial of PI(3)P Standard (K-3003) for a 4 µM stock. Vortex briefly to fully reconstitute the lipid. Spin down briefly and place vial at room temperature until use. Afterwards, store unused portion of 4 µM PI(3)P standard at -20°C for up to 1 month.
4. Add following for 2 mL of Standard Curve Buffer (SCB) containing 0.1x Reaction Buffer and 4 µM PI(3,4)P2:
   - 40 µL of 5x Reaction Buffer (K-5202)
   - 40 µL of 200 µM PI(3,4)P2 substrate from step 2

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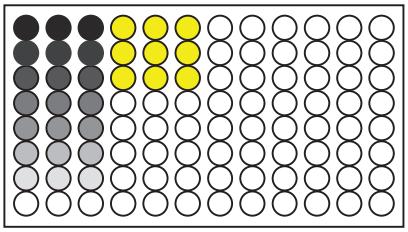
- 1.92 mL ddH2O

5. Prepare PI(3)P standards by diluting the 4 µM PI(3)P (step 11) 2-fold serially in SCB (step 12) according to the following table. Pipette up and down to mix well after each addition.

ID	PI(3)P (µM)	PI(3)P (pmol/50µL)	4 µM PI(3)P or previous dilution	Standard Curve Buffer (SCB)
A	4	200	400 µL of 4 µM (step 3)	-
B	2	100	200 µL of A	200 µL
C	1	50	200 µL of B	200 µL
D	0.5	25	200 µL of C	200 µL
E	0.25	12.5	200 µL of D	200 µL
F	0.125	6.25	200 µL of E	200 µL
G	0.0625	3.125	200 µL of F	200 µL

6. Set up detection plate (K-3001) as shown below.
   a. Add 20 µL of 5x Detection Buffer (K-3004) to each well of the detection plate.
   b. Add 50 µL of 0.1x RB to wells C4-C6 and H1-H3 as No Lipid and Blank controls respectively.
   c. Add 50 µL PI(3)P standard dilutions to wells 1-3 of rows A through G as indicated.
   d. Add 50 µL C+ to wells A4-A6 as enzyme positive control.
   e. Add 50 µL NE to wells B4-B6 as No Enzyme control.
   f. Add 50 µL of sample reactions in triplicate to remaining wells
   g. Gently shake the plate on a plate shaker for 1 min.

A. 200 pmol
B. 100 pmol
C. 50 pmol
D. 25 pmol
E. 12.5 pmol
F. 6.25 pmol
G. 3.125 pmol
H. No Lipid



shaded = PI(3)P Standards, yellow = controls, white = samples

7. Reconstitute each PI(3)P Detector (K-3305) with 0.9 mL ddH2O. Pipette up and down to resuspend. Do not vortex. Pool together (totally 3.6 mL) and mix gently before use.
   a. Add 30 µL of ddH2O to blank control wells (H1-H3).
   b. Add 30 µL of reconstituted PI(3)P detector to all wells except blank control wells (H1-H3).
8. Seal plate and incubate on plate shaker at room temperature for 1 hour. Do not prolong incubation time.
9. Dilute 20 mL 10X TBS-T buffer (K-TBST) with 180 mL ddH2O to get 200 mL TBS-T.
10. Discard solution from detection plate and wash plate 3 times briefly with TBS-T (200 µL/well).
11. Dilute Secondary Detector (K-SEC2) 1:200 with TBS-T by adding 60 µL Secondary Detector to 12 mL TBS-T and mix well. Discard TBS-T wash from plate and add 100 µL diluted Secondary Detector to each well. Seal plate and incubate for 30 min at room temperature on a plate shaker.
12. Discard solution from Detection Plate and wash plate 3 times with TBS-T (200 µL/well.)
13. Discard TBS-T wash completely from plate. Add 100 µL TMB to each well. Seal plate with a new plate seal. Allow color to develop for 30 minutes in dark at room temperature without agitation. Stop color development by adding 50 µL of 1 N H2SO4 stop solution (K-STOPt) to each well. Blue color will change to yellow color upon addition of stop solution. Eliminate any big air bubbles present in wells before reading the plate.

Caution: Be aware of the dynamic range of your plate reader instrument. Make sure the highest absorbance reading is within this range. If needed, the TMB incubation time can be shortened to accomplish this.

14. Read absorbance at 450 nm on a plate reader.

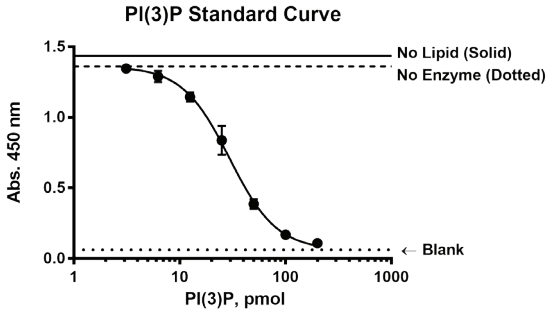
Data Analysis

Enzyme activity can be estimated by comparing the values from the wells containing enzymatic reaction products to the values in the standard curve.

Plot the absorbance values vs. Log PI(3)P in pmol to generate a standard curve using sigmoidal dose-response (variable slope), non-linear regression analysis as shown to the right. Constrain the curve bottom to Blank OD450nm and curve top to NE OD450nm. Determine the PI(3)P value in pmol for each enzymatic reaction assay point (50 µL out of 200 µL final reaction mixture) by interpolation from the standard curve. Calculate %Conversion and [PI(3)P] in the original 20 µL INPP4 reaction as follows:

1) %Conversion = _____ pmol PI(3)P ÷ 200 pmol × 100% = _____ %

2) [PI(3)P] = _____ pmol PI(3)P × (200 µL ÷ 50 µL) ÷ 20 µL = _____



Echelon Biosciences Inc.
Ph: 801-588-0455
Fax: 801-588-0497
Echelon-inc.com

References

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Related Products

Products	Catalog Number
Enzymes and Assays	
Malachite Green Assay kit PI(3,4)P2 Mass ELISA PI(3)P Mass ELISA Class III PI3K ELISA Kit	K-1500 K-3800 K-3300 K-3000
INPP4A Enzyme	E-8000
Lipids and Substrates	
PI(3,4)P2	P-3408, P-3416
Labeled PI(3,4)P2	C-34B6, C-34B6a, C-34F6a
PI(3)P	P-3004, P-3008, P-3016
Labeled PI(3)P	C-03B6, C-03B6a, C-03F6a

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Ph: 801-588-0455
Fax: 801-588-0497
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