Echelon Biosciences Inc.

PTEN Activity ELISA

K-4700 (96 tests)

Support: echelon@echelon-inc.com

Description: 96-well ELISA Assay for Detection and Quantification of PTEN Phosphatase Activity

Materials Provided

Catalog #	Description	Amount
K-4701	PI(4,5)P2 Coated Strip-well Detection Plate	1 plate
K-4702	PI(3,4,5)P3 Substrate, diC16	30 nmol
K-4703	PI(4,5)P2 Standard, diC16	12 nmol
K-2302	PI(4,5)P2 Detector	2 x 2.5 µg
K-4704	5x PTEN Reaction Buffer	4 mL
K-GS01	Protein Stabilizer	600 µL
K-SEC2	Secondary Detector	300 µL
K-PTAB	PBS Tablet	1 tablet
K-PBST3	10x PBS-T Buffer	30 mL
K-TMB1	TMB Solution	12 mL
K-STOPt	1 N H_2SO_4 Stop Solution	8 mL
K-DTT1	DTT	3 x 50 µmol
---	Yellow 96-well incubation plate	1 plate
---	Clear acetate plate sealer	3 seals

Additional Materials Provided by User

- Source of PTEN Enzyme (cat# E-3000).
- Incubated plate shaker or 37 °C incubator.
- Microplate Reader with capability to read absorbance at 450 nm.

Quick Protocol

Isolate PTEN samples and dilute using PTEN Reaction Buffer

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Set up PTEN reactions and incubate for 1-4 hours at 37°C

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Prepare the PI(4,5)P2 standards and controls and transfer to the incubation plate

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Transfer the PTEN samples to the incubation plate and incubate with the PI(4,5)P2 Detector for 1 hour at RT

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Transfer the reaction mixtures to the Detection Plate and incubate for 60 min at 37°C

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Wash the wells 3 times with 200 µL/well PBST

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Incubate with the Secondary Detector for 30 minutes at RT

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Wash the plate and develop with TMB for 15-30 minutes at RT

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Add 50 µL/well Stop Solution and read absorbance at 450 nm.

Storage

The kit comes in two parts with different storage requirements. Upon receipt store Kit Part 1 at 4 °C and Kit Part 2 at -80 °C.

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Background

PTEN (Phosphatase and Tensin Homolog deleted on Chromosome 10) is a 3' phosphoinositide phosphatase that converts PI(3,4,5)P3 to PI(4,5)P2 thus opposing PKB/Akt activation by PI 3-K. PTEN is involved in neuronal stem cell proliferation and self-renewal, cardiac myocyte hypertrophy and contractility, and a wide range of developmental processes. PTEN, however, is best known for its role as a tumor suppressor. Loss of PTEN activity results in accumulation of PI(3,4,5)P3,[1] abnormal activation of PKB/Akt, unregulated cell growth, suppression of apoptosis, and increased tumorigenesis in a number of human tissues. It has also been proposed that PTEN is a candidate for targeted chemotherapy because certain anti-cancer agents preferentially destroy tumors with PTEN mutations. In addition to this direct role in cancer, PTEN has recently been shown to regulate cancer-associated pathways including VEGF-mediated angiogenesis and others.

Assay Design

Echelon's PTEN Activity ELISA is designed to detect and quantify PTEN phosphatase activity by means of a competitive ELISA format, in which the signal is inversely proportional to the amount of PI(4,5)P2 produced, eliminating the need for radioactivity, organic solvents, and thin layer chromatography. The PTEN Activity ELISA directly detects PI(4,5)P2 compared to other assays, which detect free phosphate. This eliminates many possible sources of error due to the fact that inorganic phosphate is the product of many phosphatase enzyme activities, and is found in common buffers and cleaning products.

Assay Notes

1. Optimization of the PTEN reactions by enzyme amount, reaction time and temperature may be required.
2. The provided 5x PTEN Reaction Buffer (K-4704) must be used for the PTEN reactions.
3. Vigorous shaking will kill PTEN enzyme activity. When using PTEN bound to beads, some agitation will be necessary to keep beads in suspension. When using recombinant PTEN there is no need to shake the reaction.
4. PTEN reactions can be carried out at either 37 °C or at room temperature. When performing reactions at room temperature, the incubation time should be increased.
5. The amount of enzyme to use per PTEN reaction will vary according to your individual experiment. Whether you are using purified PTEN or enzyme immunoprecipitated from cell lysate, you will need to try reactions using different amounts of enzyme to determine the optimum condition. When using purified recombinant PTEN from Echelon (Cat# E-3000), enzyme concentration of 0.5-2 ng/µL is suggested as a starting point. In testing, we found that enzyme immunoprecipitated from cell lysate containing 1-5 mg cellular protein is usually sufficient for each reaction.
6. Beads need to be removed from enzyme reactions by centrifugation before reactions are stored or detected.
7. Step 1 of the "Detection" protocol requires an incubation at 37 °C with shaking. If an incubated plate shaker is not available, then the incubation can be done at 37 °C without shaking for a period of 2 hours.
8. The detection plate is composed of 12 of 8-well strips. Unused strip wells should be removed from the plate frame and stored in a clean sealable plastic bag at 4 °C. Save the plate frame after assay for future use of the remaining strip wells.
9. Never let the detection plate dry out after the ELISA assay has started. Always prepare the next solution needed before discarding the current solution from wells in use.

Assay Protocol

Please read this entire section and Assay Notes before beginning .

Reagent Preparation

1. PTEN Reaction Buffer
   Prepare fresh PTEN Reaction Buffer and DTT for use on the day of the assay. Dilute the 5x PTEN Reaction Buffer (K-4704) 5-fold in dH2O and supplement with 10 mM DTT (K-DTT1). Each vial of K-DTT1 contains 50 µmol DTT. Add 50 µL dH2O for a 1 M stock. Once reconstituted, the DTT should be used immediately and any remaining should be discarded.

   5 mL of PTEN Reaction Buffer = 1 mL of 5x PTEN Reaction Buffer + 50 µL 1 M DTT + 3,950 µL dH2O.

2. PBS Buffer
   Prepare the PBS Buffer by dissolving the provided PBS tablet (K-PTAB) in 200 mL dH2O.
3. PBS-T Buffer
   Prepare the 1x PBS-T Buffer by diluting 30 mL of the 10x PBS-T Buffer (K-PBST3) with 270 mL dH2O.
4. PI(3,4,5)P3 Substrate
   Equilibrate vial of PI(3,4,5)P3 Substrate (K-4702) to room temperature. Prepare a 100 µM PI(3,4,5)P3 Substrate stock solution by adding 300 µL dH2O to the vial of PI(3,4,5)P3 Substrate (K-4702). Vortex for at least 60 seconds to resuspend the lipid. Spin down and place vial at room temperature. Prior to use, dilute the required amount of PI(3,4,5)P3 Substrate in PTEN Reaction Buffer for a 16 µM (2x conc.) working solution. 1 mL of 16 µM PI(3,4,5)P3 Substrate can set up 30 PTEN reactions. The unused portion of 100 µM PI(3,4,5)P3 Substrate stock can be stored at -20 °C for up to 3 months.

   1 mL of 16 µM PI(3,4,5)P3 Substrate = 160 µL of 100 µM PI(3,4,5)P3 Substrate stock + 840 µL of PTEN Reaction Buffer.

5. PI(4,5)P2 Standard
   Equilibrate vial of PI(4,5)P2 Standard (K-4703) to room temperature. Prepare a 40 µM PI(4,5)P2 Standard stock solution by adding 300 µL dH2O to the vial of PI(4,5)P2 Standard (K-4703). Vortex for at least 60 seconds to resuspend the lipid. Spin down and place vial at room temperature. The unused portion of 40 µM PI(4,5)P2 Standard stock can be stored at -20 °C for up to 3 months.

PTEN Reaction and Incubation

1. Isolate or prepare PTEN according to usual protocols. See attached support protocol for immunoprecipitation of PTEN from cells. Prior to use, dilute the required amount of PTEN to a 2x concentration in the PTEN Reaction Buffer.
2. PTEN reactions can be set up in micro centrifuge tubes or in the yellow Incubation Plate (using recombinant PTEN enzyme only). Please read assay notes at the end of the protocol first.
   a. For each 60 µL PTEN reaction (for duplicate assay points): combine 30 µL of the 16 µM PI(3,4,5)P3 Substrate (480 pmol) and 30 µL of the PTEN (2x conc.). You may also include an Enzyme Only control by replacing 30 µL of Substrate with 30 µL of PTEN Reaction Buffer.
   b. Seal the PTEN reactions and let them proceed without vigorous shaking for a certain time, usually 1-4 hours at 37 °C or RT

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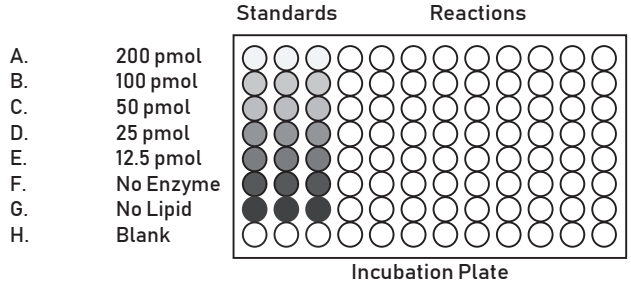
Note: If using PTEN bound to beads, light agitation or slow rotation will be necessary to keep beads in suspension.

3. Stop each 60 µL reaction.
   a. If PTEN enzyme is bound to beads, centrifuge to separate the beads and transfer reaction supernatant to clean tubes.
   b. For recombinant PTEN, heat the reaction for 3 minutes at 95 °C to stop the reaction.
4. Add an additional 62 µL of PTEN Reaction Buffer to each stopped reaction for a total of 122 µL.
   Note: Reaction products can be stored at -20 °C for up to a week. ELISA Detection can be run on another day. We suggest that standards and controls be run in duplicate or triplicate. The Incubation Plate layout shown on the next page gives an example plate layout with triplicate standards and controls.
5. Prepare PI(4,5)P2 Standards and Controls
   a. From the 40 µM PI(4,5)P2 Standard stock prepared earlier, prepare a 4 µM working solution by adding 40 µL of the 40 µM stock solution to 360 µL of the PTEN Reaction Buffer.
   b. Make four, 2-fold serial dilutions from the 4 µM PI(4,5)P2 stock with PTEN Reaction Buffer.

      e.g. Each 400 µL dilution = 200 µL previous dilution + 200 µL PTEN Reaction Buffer.

   c. Prepare a 4 µM working solution of PI(3,4,5)P3 Substrate for the "No Enzyme" control by diluting 8 µL of the 100 µM PI(3,4,5)P3 Substrate stock solution with 192 µL PTEN Reaction Buffer.
   d. Pipet 60 µL/well of each PI(4,5)P2 Standard solution to rows A through E of the Incubation Plate.
   e. Pipet 60 µL/well of the 4 µM PI(3,4,5)P3 Substrate solution to the No Enzyme control wells in row F of the Incubation Plate.
   f. Pipet 60 µL/well of PTEN Reaction Buffer to the No Lipid control wells in row G of the Incubation Plate.
   g. Pipet 120 µL/well of PTEN Reaction Buffer to the Blank control wells in row H of the Incubation Plate.

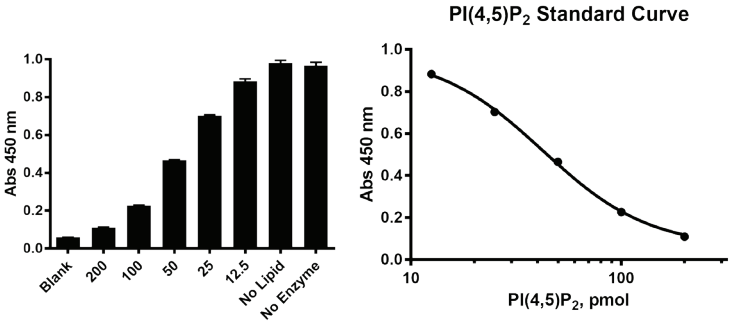


PIP2 Standards and Controls	PIP2 per 50 µL solution
4 µM	200 pmol
2 µM	100 pmol
1 µM	50 pmol
0.5 µM	25 pmol
0.25 µM	12.5 pmol
No Enzyme control	0 pmol (with Substrate)
No Lipid control	0 pmol

6. Transfer 60 µL/well of each stopped PTEN reaction into 2 wells of the Incubation Plate for duplicate data points.
7. Dilute the PI(4,5)P2 Detector to 0.25 µg/mL in Detection Buffer:
   a. Prepare 10 mL of Detection Buffer by adding 200 uL Protein Stabilizer (K-GS01) to 9.8 mL of PBS Buffer.
   b. Pipette 1 mL of Detection Buffer into a vial of PI(4,5)P2 Detector (K-2302). Mix gently to reconstitute the Detector. Spin down and transfer the 1 mL of reconstituted PI(4,5)P2 Detector into the 9 mL of Detection Buffer.
      Note: Once the PI(4,5)P2 Detector has been reconstituted in Detection Buffer, it is only good for 1 day. It cannot be stored for later use.
8. Incubate with PI(4,5)P2 Detector.
   a. Add 60 µL/well of the 0.25 ug/mL PI(4,5)P2 Detector to all Control, Standard and PTEN reaction wells except the Blank controls in row H.
   b. Seal the Incubation Plate and incubate for 60 minutes at room temperature with gentle agitation on a plate shaker.

Detection

1. Following the incubation, transfer the reacted mixtures to the Detection Plate (K-4701,clear flat-bottom strip plate). Transfer 100 µL from each well to the corresponding well in the Detection Plate. (This can easily be accomplished with a multi-channel pipettor.) Seal the plate and incubate for 60 minutes at 37 °C with agitation on a plate shaker.
2. During the incubation prepare the Secondary Detector. Briefly centrifuge the vial of Secondary Detector (K-SEC2). Dilute the Secondary Detector 1:48 with PBS-T. Dilute ONLY the amount you will use for the current assay and store the remainder of the Secondary Detector at 4 °C for future use.
3. After incubation, discard solution from the Detection Plate and wash the wells 3 times with 200 µL/well of PBS-T.
4. Add 100 µL of diluted Secondary Detector to each well of the Detection Plate. Seal the plate and incubate for another 60 minutes on a plate shaker at room temperature.
5. Discard the Secondary Detector from the Detection Plate and wash the wells 3 times with 200 µL/well of PBS-T.
6. Immediately add 100 µL of TMB solution (K-TMB1) to each well. Allow color to develop for 15-30 minutes in the dark. Watch for blue color development and DO NOT overdevelop. Stop color development by adding 50 µL of 1 N H2SO4 Stop Solution (K-STOPt) to each well when the color has turned dark ocean blue in the No Lipid control wells but is still clear to very faint blue in the 200 pmol PI(4,5)P2 Standard wells. 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: Use caution when dealing with corrosive 1 N H2SO4 Stop Solution.
7. Read absorbance at 450 nm on a plate reader.



PTEN activity can be estimated by comparing the absorbance values from the wells containing enzyme reaction products to the values in the standard curve. Plot the absorbance values obtained vs. log of PI(4,5)P2 in pmol per standard to generate a standard curve using sigmoidal dose-response (variable slope) correlation. Determine the PI(4,5)P2 level in pmol by interpolation from absorbance values

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obtained from the enzyme reactions. PTEN activity in your samples can be estimated by the percentage conversion from initial 200 pmol of PI(3,4,5)P3 per assay point.

Support Protocol

Immunoprecipitation of PTEN From Cells

The following support protocol for immunoprecipitation (IP) of PTEN from cells has been validated for use with the PTEN Activity ELISA. Further optimization of this protocol and/or different IP protocols can be used to IP PTEN from cells depending on the needs and experience of the user.

Materials Needed	Company	Catalog Number
1.5 mL Centifuge Tubes	N/A	N/A
Lysis Buffer (25 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 1mM EDTA, 5% Glycerol)	N/A	N/A
Protease Inhibitor Cocktail	Sigma	P8340
Protein A Agarose Beads	Pierce	20333
PTEN (D4.3) XP™ Rabbit mAb	Cell Signaling	9188
PTEN Reaction Buffer	EBI	K-4704
PBS	N/A	N/A

This protocol is written for a 100 mm dish of NIH 3T3 cells (90% confluent) for about 15-20 mg/mL of cellular protein. To maximize PTEN activity; keep all solutions ice cold, carry out all reactions on ice or at 4°C, and use a centrifuge that is equilibrated at 4°C.

Reagent Preparation

Lysis Buffer

Prepare the Lysis Buffer according to the recipe listed on the materials. Add the protease inhibitor cocktail fresh at a 1:100 dilution. Place buffer on ice and chill until ice cold.

PTEN Reaction Buffer

See preparation instructions in the "Reagent Preparation" section in the main protocol. PTEN Reaction Buffer must be made fresh just prior to reaching step 6 in the IP protocol. Keep buffer at room temperature once prepared.

Cell Lysis

1. Place cell culture dish on ice and wash cells twice with ice cold PBS.
2. Add 0.5 mL of ice cold Lysis Buffer to cells. Scrape cells and transfer mixture into a cooled 1.5 mL centrifuge tube.
3. Incubate cells for 15 minutes with constant agitation at 4°C.
4. Centrifuge cells for 10 minutes at 14,000 x g to pellet cells.
5. Transfer supernatant to a fresh, cooled 1.5 mL centrifuge tube and place on ice. Discard pellet.

Immunoprecipitation

1. Transfer 400 µL of the cell lysate to a fresh, cold, 1.5 mL centrifuge tube.
2. Add 8 µL of the anti-PTEN antibody (Cell Signaling) to the lysate. Incubate 3 hours to overnight at 4°C with agitation.
3. Add 60 µL of the 50% Protein A agarose beads to the mixture and incubate 2-3 hours at 4°C.
4. Briefly centrifuge to pellet beads. Discard the supernatant.
5. Wash the bead complex three times with ice cold PBS. Centrifuge and discard solution after each wash.
6. Wash once with PTEN Reaction Buffer.
7. Resuspend bead complex in 30 µL of PTEN Reaction buffer.
8. Proceed immediately with the PTEN reactions by adding 30 µL of the 16 µM PI(3,4,5)P3 Substrate to the bead complex. (See the "PTEN Reaction and Incubation" section in the main protocol).

References

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Related Products

Catalog #	Products
Assays	
K-2500s	PIP3 Mass ELISA
K-4500	PIP2 Mass ELISA
K-1000s	PI3-Kinase Activity ELISA: Pico
K-1500	Malachite Green Assay Kit
PTEN Inhibitors	
B-0350	SF1670
B-0351	VO-OHpic
Enzymes and Substrates	
E-3000	PTEN enzyme, active
PI(3,4,5)P3	P-3908, P-3916
PI(4,5)P2	P-4508, P-4516

Please visit our website at www.echelon-inc.com for more enzyme and lipid products.

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