Chapter 6

Assays to Measure PTEN Lipid Phosphatase Activity In Vitro from Purified Enzyme or Immunoprecipitates

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Abstract

PTEN is a one of the most frequently mutated tumor suppressors in human cancers. It is essential for regulating diverse biological processes and through its lipid phosphatase activity regulates the PI 3-Kinase signaling pathway. Sensitive phosphatase assays are employed to study the catalytic activity of PTEN against phospholipid substrates. Here we describe protocols to assay PTEN lipid phosphatase activity using either purified enzyme (purified PTEN lipid phosphatase assay) or PTEN immunopurified from tissues or cultured cells (cellular IP PTEN lipid phosphatase assay) against vesicles containing radiolabeled PIP_3 substrate.

Key words PTEN, Phosphoinositide, Phosphatase, Phosphate, Tumor suppressor, PI3-Kinase, Cancer

1 Introduction

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a phosphatase that mainly localizes in the cytoplasm and antagonizes the phosphoinositide 3-kinase (PI3K) signaling pathway by dephosphorylating plasma membrane localized phosphatidylinositol-3,4,5-trisphosphate (PIP_3) and negatively regulating its downstream targets [1]. PTEN has been heavily studied due to its status as a tumor suppressor gene in which loss of function mutations are identified in many sporadic tumors and in the germ line of patients with Cowden disease and related phenotypes [2–6]. The predominant product of the *PTEN* gene is a 403-amino acid protein structured with an N-terminal phosphatase domain (residues 7–185) and a C-terminal C2 domain (residues 186–351). These two domains constitute the minimal catalytic unit [7, 8]. The phosphatase domain contains the highly conserved HCXXGXXR(S/T) (CX5R) active site motif, hallmark of protein tyrosine phosphatases [9], with Cysteine 124 required for catalysis as the active site nucleophile, and with many different mutations to

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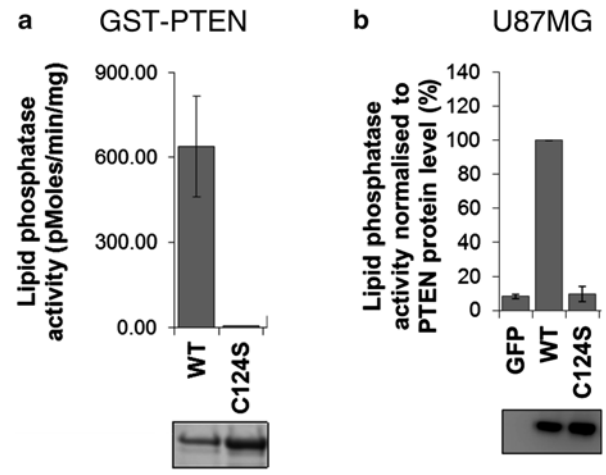

Fig. 1 PTEN assay data. Example experiments following the described protocol are shown using either (**a**) GST-PTEN (1 h and 500 ng enzyme at 30 °C assaying either wild-type enzyme or the phosphatase inactive mutant PTEN C124S) or (**b**) Untagged recombinant human PTEN protein was immunoprecipitated from 1 mg soluble protein extract derived from PTEN null U87MG cells. In the latter case, cells were transduced with viruses encoding either GFP, wild-type PTEN or PTEN C124S. Western blots using anti-PTEN antibodies display the amount of PTEN protein in each assay. This figure is adapted from data previously published [6]

the phosphatase domain resulting in loss of the phosphatase activity. As a lipid phosphatase, it is known that PTEN antagonizes PI3-Kinase, dephosphorylating at the D3 position, the PI3K lipid product PtdIns(3,4,5)P_3, which is generated by PI3K phosphorylation of PtdIns(4,5)P_2 [10, 11]. Through this mechanism PTEN affects AKT/PKB and other PIP_3-regulated proteins and thus a number of cell biological processes regulated by PI3K via PIP_3. Assaying PTEN lipid phosphatase activity in vitro is therefore a starting point to study PTEN function [12, 13]. Here, we describe in vitro protocols and present example data (Fig. 1), studying the catalytic activity of PTEN against phospholipid vesicle substrates containing radiolabeled PIP_3 that in our experience can give substantially greater sensitivity than assays detecting phosphate release using malachite green reagent and can detect low ng quantities of input enzyme.

2 Materials

2.1 Production of 3-(^{33}P)-PtdIns (3,4,5)P_3

1. PtdIns(4,5)P_2. Prepare 1 mM stock in methanol–chloroform 1:1. Store at −20 °C (*see* **Note 1**).
2. Phosphatidylserine (PS). Prepare a 5 mM stock in: chloroform–methanol 1:1. Store at −20 °C.

3. Resuspension buffer: 25 mM Hepes pH 7.4, 100 mM NaCl, and 1 mM EGTA.
4. γ-^{33}P ATP. Stored at −20 °C following radioisotope guidelines.
5. PI3K α (p110α and p85α heterodimer co-expressed and purified from insect cells or commercially available) and stored in small aliquots at −80 °C.
6. Reaction buffer: 25 mM Hepes pH 7.4, 100 mM NaCl, 1 mM EGTA, 0.2 mM EDTA, 2.5 mM $MgCl_2$, 1 mM DTT, 1 mM sodium orthovanadate, 0.2 mM PMSF, 1 mM benzamidine (*see* **Note 2**).
7. Assay termination buffer: chloroform–methanol–HCl (40:80:1) (*see* **Note 3**).
8. Chloroform ($CHCl_3$).
9. 0.1 M HCl.
10. Synthetic upper phase (*see* **Note 4**).
11. Phosphorylated substrate resuspension solvent: chloroform–methanol (2:1 v/v) (*see* **Note 5**).
12. Cup horn bath sonicator.
13. Centrifuge vacuum dryer.
14. Scintillation cocktail.
15. Liquid scintillation counter.

2.2 In Vitro PTEN Lipid Phosphatase Assay Components

1. Phosphatidylcholine (PC). Prepare a 33 mM stock in: chloroform–methanol 1:1. Store at −20 °C.
2. diC16 PtdIns(3,4,5)P_3. Prepare 1 mM stock in methanol–chloroform 1:1. Store at −20 °C (*see* **Note 6**).
3. ^{33}P-PtdIns(3,4,5)P_3 prepared and stored as described.
4. Lipid Vesicle resuspension buffer: 10 mM Hepes pH 7.4, 1 mM EGTA.
5. Purified recombinant PTEN protein (usually 100 ng per assay) in buffer containing: 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 10% glycerol. Store in small aliquots at −80 °C (*see* **Note 7**).
6. Reaction buffer: 50 mM Hepes pH 7.4, 150 mM NaCl, 1 mM EGTA, 10 mM DTT.
7. Bovine serum albumin (BSA) (10 mg/ml) (*see* **Note 8**).
8. 1 M perchloric acid (PCA) (*see* **Note 9**).
9. 10% (v/v) ammonium molybdate (*see* **Note 10**).
10. Toluene–isobutyl alcohol (1:1 v/v) (*see* **Note 11**).
11. Scintillation cocktail.

12. Centrifuge vacuum dryer.
13. Cup horn bath sonicator.
14. Scintillation counter.

2.3 Immunoprecipitated Cellular PTEN Lipid Phosphatase Assay Components

1. Tissue or cultured mammalian cells expressing PTEN.
2. Cell lysis buffer: 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 50 mM NaF, 1 % NP-40 and containing freshly added 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, and 0.1 % β-mercaptoethanol.
3. 1× phosphate-buffered saline (PBS).
4. Protein G sepharose FastFlow beads (Sigma-Aldrich, St. Louis, MO, USA) (*see* **Note 12**).
5. Washing buffer: 50 mM Tris–HCl pH 7.4, 300 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 50 mM NaF, 1 % NP-40 and containing freshly added 0.2 mM PMSF, 1 mM benzamidine, and 0.1 % β-mercaptoethanol.
6. Anti-PTEN antibody, e.g., Mouse monoclonal A2B1 unconjugated (Santa Cruz Biotechnology, Dallas, Texas, USA).
7. Phosphatidylcholine (PC) (Avanti Polar Lipids, Inc.). Prepare a 33 mM stock in: chloroform–methanol 1:1. Store at –20 °C.
8. diC16 PtdIns(3,4,5)P_3 (Cellsignals, Columbus, OH). Prepare 1 mM stock in methanol–chloroform 1:1. Store at –20 °C (*see* **Note 6**).
9. ^{33}P-PtdIns(3,4,5)P_3 prepared as described (*see* Subheading 3.1).
10. Lipid Vesicle resuspension buffer: 10 mM Hepes pH 7.4, 1 mM EGTA.
11. Reaction buffer: 50 mM Hepes pH 7.4, 150 mM NaCl, 1 mM EGTA, 10 mM DTT.
12. BSA (10 mg/ml) (*see* **Note 8**).
13. 1 M PCA (*see* **Note 9**).
14. 10 % (v/v) ammonium molybdate (*see* **Note 10**).
15. Toluene–isobutyl alcohol (1:1 v/v) (*see* **Note 11**).
16. Scintillation cocktail.
17. Centrifuge vacuum dryer.
18. Cup horn bath sonicator.
19. Liquid scintillation counter.
20. 4× lithium dodecyl sulfate (LDS) sample loading buffer.
21. β-mercaptoethanol.
22. 4–12 % precast SDS-polyacrylamide gel (SDS-PAGE) (*see* **Note 13**).

23. Polyvinylidene difluoride (PVDF) membrane.
24. PTEN antibody for immunoblotting. Several good antibodies are commercially available and the use of an antibody raised in a different species may allow immunoblotting for PTEN without interference from secondary antibody recognition of the primary anti-PTEN antibody originally used in immunoprecipitation.

3 Methods

Carry out all procedures on ice unless otherwise specified. Work with extreme care: remember you are using radioisotopes (*see* **Note 14**).

3.1 Production of 3-(^{33}P)-PtdIns(3,4,5)P_3 Substrate by Labeling at the D3 Position

1. Plan the scale of the substrate preparation required. Here we describe a 100 µl PI3K reaction but this could be scaled up or down. To a 2 ml screw cap Eppendorf tube add sufficient volume of lipid stocks (PS and PtdIns(4,5)P_2) that will give a final concentration of 100 µM phosphatidylserine (PS—2 µl from 5 mM stock) and 100 µM PtdIns(4,5)P_2 (10 µl from 1 mM stock) in the final total volume of aqueous reaction mix.
2. Dry the lipid mixture under vacuum to remove all organic solvents (*see* **Note 15**).
3. Submerge the lipid film in 50 µl of Lipid Reaction Buffer (LRB): 25 mM Hepes pH 7.4, 100 mM sodium chloride and 1 mM EGTA.
4. Sonicate the lipid mixture using a cup horn sonicator by three cycles of 15 s to resuspend the lipids, forming heterogeneous vesicles.
5. To the lipid vesicles add 25 µl of Kinase Buffer (25 mM Hepes pH 7.4, 100 mM sodium chloride, 1 mM EGTA, 0.2 mM EDTA, 2.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM benzamidine) 500 µCi of (γ-^{33}P) ATP (18.5 MBq), and 10 µg of the PI3K to a total of 100 µl.
6. Incubate the mixture for 45 min in a water bath at 37 °C.
7. Add a second aliquot of PI3K and incubate for further 45 min at 37 °C.
8. Terminate the reaction by adding 750 µl of $CHCl_3$–CH_3OH–HCl (40:80:1).
9. Add 250 µl of chloroform and 400 µl 0.1 M HCl to make the ratio $CHCl_3$ (1)–CH_3OH (1)–aqueous (0.9).

10. Two layers will form. The sequential addition of solvent components is to allow acidification in a single phase before the phase split.
11. Remove and discard the all aqueous upper phase and any interphase precipitate (*see* **Note 16**).
12. Wash the lower organic phase three times with synthetic upper phase (*see* **Note 17**).
13. Dry the lower phase in a vacuum dryer (*see* **Note 18**).
14. Resuspend the dried substrate in 500 µl of $CHCl_3$–CH_3OH (2:1 v/v).
15. Add 2 µl of each of the three washes and 2 µl of substrate to individual tubes containing the scintillation cocktail and mix.
16. Determine the radioisotope incorporation into the ^{33}P labeled substrate by using a scintillation counter. Using the initial specific activity of the ATP, the concentration of ^{33}P-PIP_3 can be estimated.
17. Store the radiolabeled substrate at −20 °C following appropriate radioisotope guidelines.

3.2 In Vitro PTEN Lipid Phosphatase Assay

1. Determine the total volume of assay mix required for the day's experiments, using a volume of for example 100 µl per assay and with some residual.
2. Lipid vesicles are prepared by mixing sufficient lipid stocks to give a final concentration of 100 µM of phosphatidylcholine (PC—from 33 mM stock), 1 µM of diC16 PtdIns(3,4,5)P_3 in the final assay mixture along with a volume of ^{33}P-PtdIns(3,4,5)P_3 to give 100,000 dpm counts per assay (*see* **Note 19**).
3. Dry the lipids using a centrifuge vacuum dryer.
4. Submerge the dried substrate in a buffer containing 10 mM Hepes pH 7.4 and 1 mM EGTA.
5. Sonicate the mixture in a cup horn sonicator for three 15 s bursts, followed by 5 min of ice incubation to prepare lipid vesicles.
6. Dilute the enzyme (usually 100 ng (2 pmol) of recombinant PTEN per assay) in 50 µl of H_2O (*see* **Note 20**).
7. Add 25 µl of assay buffer containing 50 mM Hepes pH 7.4, 1 mM EGTA, 10 mM dithiothreitol (DTT) and 150 mM NaCl.
8. Add 25 µl of the radiolabeled substrate vesicles.
9. Incubate for 1 h or, in case of a time course, for the respective time points, at 37 °C on a shaker (*see* **Note 21**).
10. Terminate the reaction through the addition of 10 µl of BSA (10 mg/ml) and 500 µl of 1 M ice-cold PCA.

11. Mix the samples by vortex and incubate for 30 min on ice.
12. Centrifuge the samples at 14,000×*g* for 10 min at 4 °C to remove precipitated lipid and protein and transfer the supernatants to new tubes.
13. Add 10% (w/v) ammonium molybdate to the supernatant to form a complex with free phosphate released during the assay, which will become soluble in the later organic phase. Vortex and incubate for 10 min at room temperature.
14. Add 1 ml toluene–isobutyl alcohol (1:1 v/v). A two-phase mix will form.
15. Remove 500 μl of the upper organic phase containing the phosphate complex and mix it into 8 ml of liquid scintillation cocktail.
16. Determine the radioactivity using a liquid scintillation counter.

3.3 In Cell PTEN Lipid Phosphatase Assay

1. Seed cells, e.g., U87MG (PTEN null) or HEK293T cells (highly transfectable), in 10 cm dishes in 10 ml of recommended medium. Grow cells in a 37 °C incubator with 5% CO_2.
2. If necessary, express wild-type PTEN protein or mutants using plasmids transfection or lentiviral particles.
3. Immediately prior to lysis, wash the cells twice with 5 ml ice-cold 1× PBS.
4. Lyse cells on ice using 1 ml cell lysis buffer per 10 cm dish (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 50 mM NaF, 1% NP-40 and containing freshly added 0.2 mM PMSF), 1 mM benzamidine. Rotate the dish carefully to let the lysis buffer cover the entire area of grown cells.
5. Scrape the cells on ice with a cell scraper. Collect and transfer the cell lysates into a 1.5 ml microfuge tube on ice. Incubate the lysate on ice for 30 min.
6. Centrifuge the samples at 14,000×*g* for 15 min at 4 °C to remove any aggregated insoluble proteins and cellular debris.
7. Transfer the supernatant to a new ice-cold microfuge tube and keep on ice. Be careful not to transfer any pellet.
8. While the cell lysates are incubating on ice for 30 min, cut the narrow end of a P-200 pipette tip and transfer an appropriate amount of 50% protein G sepharose bead slurry (10 μl per sample) to a 1.5 ml microfuge tube (*see* **Notes 12** and **22**). Wash the beads thrice with 200 μl ice-cold PBS by centrifuging at 1000×*g* for 1 min.
9. Resuspend the beads in 500 μl of lysis buffer. Pre-couple the protein G sepharose beads with the antibody by incubating it

with PTEN A2B1 antibody at 4 °C for 1 h while gently mixing on a rotating shaker. Centrifuge the antibody-coupled beads at 2000 × *g* for 1 min at 4 °C and discard the supernatant. Pipette 10 μl of PTEN-antibody coupled beads to new Eppendorf tubes, one for each sample.

10. Add 1 ml of cell lysate to the PTEN-antibody coupled beads and incubate at 4 °C for 1 h with constant rotation.
11. Centrifuge the beads at 2000 × *g* for 1 min.
12. Wash the beads twice in lysis buffer with high salt (300 mM NaCl) and then with reaction buffer (50 mM Hepes pH 7.4, 1 mM EGTA, 10 mM dithiothreitol (DTT), and 150 mM NaCl) (*see* **Note 23**).
13. Aspirate the buffer and proceed for the assay as described above.
14. Together with cell lysate used for PTEN immunoprecipitation and assay, samples should be analyzed by western blotting in order to be able to monitor the level of PTEN expression.
15. For the samples to use for western blotting analysis, wash the beads thrice using centrifuge tube filters (*see* **Note 24**).
16. Add 30 μl of 1× LDS sample loading buffer and leave for 10 min at room temperature.
17. Spin down the samples. Discard the filters and add 10% β-mercaptoethanol to the samples.
18. Heat the samples at 70 °C for 10 min.
19. Resolve the samples on 4–12% SDS-PAGE gel.
20. After electrophoresis, transfer the proteins from a gel to a PVDF membrane, for 1 h at 30 V, in the presence of running buffer, using a tank wet transfer system and following standard protocols and manufacturer's guidelines.
21. Dry the PVDF membrane for 30 min and then block it with a solution of 5% (w/v) low fat milk protein or 5% (w/v) BSA made up in TBS-T (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 2.5 mM KCl, 0.1% (v/v) Tween 20).
22. Wash the PVDF membrane for 30 min with three buffer changes of TBS-T, before its overnight incubation at 4 °C with a PTEN primary antibody made up in 5% (w/v) low fat milk protein or 5% (w/v) BSA in TBS-T.
23. Remove the primary antibody solution and wash the membrane for 30 min including three buffer changes of TBS-T.
24. Incubate the membrane with the appropriate HRP-linked secondary antibodies made up in 5% (w/v) low fat milk in TBS-T, for 1 h at room temperature.

25. Unbound secondary antibody is removed by washing the membrane over 30 min with three buffer changes of TBS-T.
26. Detect protein-antibody complexes using HRP chemiluminescence detection reagents following manufacturer' protocols (*see* **Note 25**).

4 Notes

1. The lipids used are available either in the acid form (Cellsignals, PtdIns(4,5)P_2-diC16 catalog number 902) or sodium salt form (PtdIns(4,5)P_2-diC16-Na catalog number 202). We recommend the latter.
2. Prepare the reaction buffer fresh each time. Use frozen aliquots of 1 M DTT. Add vanadate, PMSF, benzamidine, and DTT just before starting.
3. $CHCl_3$–CH_3OH–HCl (40:80:1) buffer can be made and stored at room temperature for short periods (weeks–months) in well-sealed glass containers. Evaporation of Chloroform during prolonged storage can change the ratio. Use glass pipettes or measuring cylinders to measure the solvents.
4. Synthetic upper phase refers to the upper phase solvent mixture after the phase split procedure and is prepared without dissolved solutes as a washing reagent. For precise replication of the synthetic upper phase solvent preparation,: use a 2 l separating funnel to mix methanol–chloroform–12 M HCl in a ratio 200:100:1 (750 ml final volume). Add 250 ml chloroform and 450 ml 0.1 M HCl. Mix thoroughly and leave to clarify overnight (shorter times tend to give a slightly cloudy phase split). Drain off the upper and lower phase separately and store separately at room temperature.
5. $CHCl_3$–CH_3OH (2:1 v/v) buffer can be made and stored at room temperature. Do not keep it for too long because evaporation of the components can change the ratio.
6. Cellsignals, sodium salt form (PtdIns(3,4,5)P_3-diC16-Na catalog number 208).
7. Recombinant PTEN protein can be expressed in E. coli cells as a fusion protein with glutathione *S*-transferase (GST). GST-PTEN is immobilized on GSH-Sepharose 4B bead slurry. Cleavage of the GST-tag from the fusion PTEN protein is then obtained by using PreScission protease enzyme. Add 10% glycerol to the untagged purified PTEN protein aliquots, snap-freeze in liquid nitrogen and store the aliquots at –80 °C.
8. We use essentially fatty acid free BSA prepared in water and stored at 4 °C.

9. Prepare 1 M PCA and store at 4 °C.
10. Prepare ammonium molybdate 10% w/v fresh every time. Vortex.
11. Prepare toluene–isobutyl alcohol (1:1 v/v) stock and store it at room temperature in a well-sealed glass bottle.
12. Resuspend the protein G sepharose beads thoroughly before dispensing.
13. Before loading the samples onto the Precast SDS-PAGE gels rinse the gels with tap water and gel wells at least three times with 1× running buffer.
14. Work in a designed area following the rules agreed by your institution. Always monitor the area of work, pipettes, equipment used, and yourself. If spillage occurs, clean and monitor the area of interest. Collect all the waste, liquid and/or solid, in a specific container.
15. The time required for the mixture to dry will vary based on the volume.
16. Be careful in removing the upper phase and make sure you avoid contamination of the lower phase. Alternatively, the lower phase can be removed from below the upper- and interphases to a fresh tube.
17. Add 2× lower phase volume of synthetic upper phase and leave for 5 min. Two layers will form. Keep 2 μl from each wash for radioactivity determination.
18. The time required for the mixture to dry will vary based on the volume.
19. Calculate the finale volume based on 100 μl for each sample (e.g., 100 μl × *N*—number of samples). Always prepare a sample where the enzyme is substituted with water. This will represent the assay background. If analyzing the activity of expressed PTEN mutant proteins always have in your assay a PTEN wild-type (positive control) and an inactive mutant, e.g., PTEN C124S (catalytically dead—negative control).

    The addition of a known excess concentration of non-radiolabeled PIP_3 provides a more confident substrate concentration and allows the more accurate analysis of reaction kinetics.
20. Keep proteins always on ice in order to preserve their catalytic activity.
21. If you are performing a phosphatase assay in a time point format, start from the longest time point and in a countdown manner add the substrate just before the beginning of the time point.
22. Remember to prepare a sample where the beads are incubated only with lysis buffer instead of cell lysate. This will represent

the assay background. Samples immunoprecipitated from cells lacking PTEN allow the assessment of any apparent activity derived from other contaminating phosphatases.

23. We use high salt (0.3 M NaCl) washing buffer so that nonspecific binding to the beads is reduced and thus likely to be specific to PTEN. Wash the beads at least three times to reduce background signals.
24. Use a bench centrifuge or, if available, a vacuum manifold.
25. Use X-ray film processed using an automatic developer or acquire the image directly using an imaging system.

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