Chapter 14

Methods in the Study of PTEN Structure: X-Ray Crystallography and Hydrogen Deuterium Exchange Mass Spectrometry

Glenn R. Masson, John E. Burke, and Roger L. Williams

Abstract

Despite its small size and deceptively simple domain organization, PTEN remains a challenging structural target due to its N- and C-terminal intrinsically disordered segments, and the conformational heterogeneity caused by phosphorylation of its C terminus. Using hydrogen/deuterium exchange mass spectrometry (HDX-MS), it is possible to probe the conformational dynamics of the disordered termini, and also to determine how PTEN binds to lipid membranes. Here, we describe how to purify recombinant, homogenously dephosphorylated PTEN from a eukaryotic system for subsequent investigation with HDX-MS or crystallography.

Key words HDX-MS, Mass spectrometry, Insect cell expression, Protein purification, Liposome preparation

1 Introduction

The high-resolution structure of PTEN's dual-specificity phosphatase domain and C2 domain was determined by X-ray crystallography some time ago [1]. This has proved to be an excellent resource, providing insight into the mechanism of substrate catalysis [2, 3], the individual roles of specific residues in the active site [4], and also the possible mechanisms of oncogenic mutations in PTEN [1, 5]. However, in order to facilitate the crystallization of PTEN, a construct was used that removed intrinsically disordered segments of the protein, such as the N-terminal PIP_2 binding domain, and a 50 amino acid C-terminal region. Both of these segments are critical to the regulation and function of PTEN, dictating PIP_2 binding [6, 7] and membrane localization [8]. Furthermore, the recent discovery of PTEN-Long [9–11], a variant of PTEN that is expressed with a 176 amino acid intrinsically disordered [12] N-terminal extension, has highlighted the necessity for techniques

Leonardo Salmena and Vuk Stambolic (eds.), *PTEN: Methods and Protocols*, Methods in Molecular Biology, vol. 1388, DOI 10.1007/978-1-4939-3299-3_14,

that are capable of shedding light on these areas of intrinsic disorder.

Hydrogen deuterium exchange mass spectrometry (HDX-MS) is a technique that is capable of determining structural and conformational information on proteins that harbor areas of intrinsic disorder [13]. We have used HDX-MS extensively to determine the mechanism of membrane binding for phosphatidylinositol 3-kinases (PI3Ks), and how oncogenic mutations in these enzymes induce conformational shifts that lead to an increase in kinase activity [14]. HDX-MS can also be used to determine the sites of protein:protein interactions, such as the interaction of p110β and the Gβγ subunit [15], and the site of Rab11 binding to PI4KIIIβ [16], and also has been used to identify areas of intrinsic disorder within proteins that can be subsequently removed to facilitate their crystallization [16].

Here we describe how to purify recombinant PTEN expressed in a eukaryotic system, and then use phosphatases and kinases to produce homogenously dephosphorylated or phosphorylated material. We also detail how to use HDX-MS on this material to determine the membrane-binding footprint of PTEN.

2 Materials

For either X-ray crystallography or HDX-MS, or indeed any structural biology method, it is necessary to obtain purified proteins. Although expression of recombinant PTEN has been carried out in bacteria, our experience indicates that the material obtained from baculovirus expression in Sf9 cells produces correctly folded, soluble protein of a reasonable yield (typically 0.5 mg per liter of cells). All buffers were prepared with ultrapure water and analytic grade reagents. All buffers used in the purification of PTEN were chilled to 4 °C prior to use.

2.1 PTEN Purification

1. 1 M Tris–HCl pH 8.0: Dissolve 121.14 g of Tris–HCl in 100 ml of water in a glass beaker. Adjust the pH to 8.0 (room temperature) using HCl. Bring to 1 l with water. Filter through a 0.22 μm Corning filter.
2. 5 M NaCl: Dissolve 292.2 g of NaCl in 1 l of water. Filter through a 0.22 μm Corning filter.
3. 1 M Imidazole: Dissolve 68.07 g of imidazole in 200 ml of water. Adjust the pH to 8.0 using HCl. Add water to a volume of 1 l. Filter through a Corning 0.22 μm filter.
4. 1 M HEPES pH 7.4: Dissolve 238.3 g of HEPES in 200 ml of water. Adjust the pH to 7.4 (RT) using NaOH. Add water to a volume of 1 l. Filter through a 0.22 Corning μm filter.

5. Lysis buffer: 20 mM Tris–HCl pH 8.0 (RT), 300 mM NaCl, 10 mM imidazole pH 8.0, 5 % glycerol, 2 mM β-mercaptoethanol, 0.5 % Triton X-100 (from a 10 % stock solution), with one complete EDTA-free protease inhibitor (Roche) tablet added per 50 ml of lysis buffer.
6. HisTrap buffer A: 20 mM Tris–HCl pH 8.0, 300 mM NaCl, 10 mM imidazole pH 8.0, 5 % glycerol, 2 mM β-mercaptoethanol.
7. HisTrap buffer B: 20 mM Tris–HCl pH 8.0, 100 mM NaCl, 200 mM imidazole pH 8.0, 5 % glycerol, 2 mM β-mercaptoethanol.
8. Dialysis buffer: 20 mM Tris–HCl pH 8.0, 200 mM NaCl, 5 % glycerol, 2 mM TCEP.
9. Dilution buffer: 20 mM Tris–HCl pH 8.0, 10 % glycerol, 1 mM DTT.
10. Ion exchange buffer A: 20 mM Tris–HCl pH 8.0, 50 mM NaCl, 10 % glycerol, 1 mM DTT.
11. Ion exchange buffer B: 20 mM Tris–HCl pH 8.0, 1 M NaCl, 10 % glycerol, 1 mM DTT.
12. Gel filtration buffer: 20 mM HEPES pH 7.4, 200 mM NaCl, 2 mM TCEP.
13. TEV Protease (Life Technologies).
14. 5 ml HisTrap HP (GE Healthcare Life Sciences).
15. 5 ml HiTrap Q HP (GE Healthcare Life Sciences).
16. HiLoad 16/60 Superdex 75 Gel Filtration Column (GE Healthcare Life Sciences).
17. SnakeSkin™ Dialysis Tubing, 3.5 K MWCO (Thermo Scientific).
18. ÄKTA Purification System (GE Healthcare Life Sciences).
19. Amicon® Ultra 15 ml 10 K MWCO Concentrators (Millipore).
20. Lambda Protein Phosphatase (New England Biolabs).
21. CK2 Protein Kinase (New England Biolabs).
22. GSK3β Protein Kinase (New England Biolabs).
23. 0.1 M ATP stock solution (pH 8.0).
24. Probe sonicator (such as a Sonics Vibra-Cell VCX 500).

2.2 Liposome Production

1. 10 mg/ml Porcine Brain L-α-phosphatidylethanolamine (Avanti Polar Lipids, Inc) in chloroform (*see* **Note 1**).
2. 10 mg/ml Porcine Brain L-α-phosphatidylserine (sodium salt) (Avanti Polar Lipids, Inc).
3. 10 mg/ml Porcine Brain L-α-phosphatidylcholine (sodium salt) (Avanti Polar Lipids, Inc).

4. 1 mg/ml Porcine Brain L-α-phosphatidylinositol-4,5-bisphosphate (Avanti Polar Lipids, Inc).
5. 10 mg/ml Cholesterol (Avanti Polar Lipids, Inc).
6. 10 mg/ml Porcine Brain Sphingomyelin (Avanti Polar Lipids, Inc).
7. Liquid Nitrogen.
8. A 43 °C water bath.
9. A vacuum desiccator.
10. A bath sonicator.
11. A vortex mixer.
12. Parafilm®.
13. Hamilton® Gastight Syringe, Model 1002 (Volume 2.5 ml).
14. Whatman® Anontop ten Inorganic Membrane Filters (0.1 μm pore size).
15. 0.2 M Stock solution of EGTA, pH 8.0: Dissolve 3.8 g of EGTA in 10 ml of ultrapure water. Adjust the pH of the solution to pH 8.0 using a concentrated NaOH solution. Bring to 50 ml with ultrapure water.
16. 1 M KCl stock solution: Dissolve 74.55 g of KCl in 1 l of ultrapure water.
17. Lipid Buffer: 20 mM HEPES pH 7.5, 100 mM KCl, 1 mM EGTA (0.22 μm filtered) (*see* **Note 2**).

2.3 Membrane-Binding Hydrogen Deuterium Exchange Sample Components

1. Corning® Costar® Spin-X® centrifuge tube filters (0.22 μm pore size).
2. Deuterium Oxide, 99.8 % atom D (Arcos Organics, New Jersey, USA).
3. D_2O buffer solution: 10 mM HEPES pH 7.5, 50 mM NaCl, 2 mM TCEP (with the stock solutions of HEPES, NaCl and TCEP diluted in D_2O rather than ultrapure water).
4. H_2O buffer solution: 10 mM HEPES pH 7.5, 50 mM NaCl, 2 mM TCEP (with the stock solutions of HEPES, NaCl, and TCEP diluted with ultrapure water).
5. Quench buffer: 2 M guanidinium chloride, 2.4 % formic acid. This should be made fresh, and kept on ice (dilute 1.91 g of guanidinium chloride, add 240 μl of formic acid, and make up to 10 ml with ultrapure water).
6. Purified dephosphorylated PTEN.
7. Liposomes (5 % brain phosphatidylinositol [4, 5] bisphosphate, 20 % brain phosphatidylserine, 45 % brain phosphatidylethanolamine, 15 % phosphatidylcholine, 10 % cholesterol, and 5 % sphingomyelin (all percentages given as weight/volume)).

8. Lipid buffer (20 mM HEPES pH 7.5, 100 mM KCl, 1 mM EGTA (0.22 μm filtered)).
9. Gel filtration buffer (20 mM HEPES pH 7.4, 200 mM NaCl, 2 mM TCEP).
10. Liquid nitrogen.
11. A timer.
12. 500 μl Eppendorf tubes.

2.4 HDX-MS Equipment and Solvents

1. Climate-controlled UPLC system: We have built a fluidics system and placed this into an ice box. The commercially available HDX Manager, available with the SYNAPT® G2-Si HDMS system by Waters is another possibility. Both a dual gradient analytical pump and an isocratic pump are required.
2. Poroszyme® Immobilized Pepsin Cartridge (Applied Biosystems (Life)).
3. Acquity 1.7 μm particle, 100 mm × 1 mm C18 UPLC Column (Waters).
4. Mass Spectrometer equipped with an electrospray ionization source (we have used a Waters Xevo or a Thermo Scientific Orbitrap).
5. HDX-MS Software (we have used HDExaminer (Sierra Analytics), DynamX (Waters) is another possibility).
6. Access to database search software in order to identify peptide sequences (we use Mascot Database Search (Matrix Science)).
7. 0.1 % Formic acid solution (produced with HPLC grade water).
8. 100 % Acetonitrile (HPLC Grade).

3 Methods

This protocol is for the purification of a Sf9 cell expressed, TEV-cleavable N-terminally His-tagged PTEN construct using an ÄKTA Purification System. Wherever possible the chromatography should be carried out at 4 °C, using chilled buffers, and protein-containing fractions should be kept on ice. For X-ray crystallography, the same purification method should be employed, but the construct purified should be the multiply truncated version as used by Lee et al. [1]. Subsequent information on suitable crystallisation conditions etc. can also be found in the Lee et al. manuscript materials and methods.

3.1 PTEN Purification

1. Remove the 1-8 l Sf9 cell pellet from storage at –80 °C and place within a glass beaker on ice. Add lysis buffer to the cell pellet, typically 50 ml of lysis buffer for 1 l of Sf9 cells being purified. Throughout the purification, care should be taken

that any reducing agents are added freshly to buffers, as PTEN has the propensity to become inactivated via oxidation [17]. Sonicate the cell pellet on ice with a probe sonicator, until the pellet has completely broken apart and the cells are lysed, typically for 6 min, with 10 s bursts of sonication followed by 10 s of rest to prevent the cell lysate from overheating.

2. Place the cell lysate in an ultracentrifugation vial and centrifuge at 140,000 × *g* for 45 min at 4 °C.
3. Equilibrate a 5 ml HisTrap FF column in HisTrap Buffer A by passing 20 ml of HisTrap Buffer A over the column at 4 ml/min.
4. Remove the supernatant (the soluble fraction) from the ultracentrifugation vial, and filter through a 0.45 μm filter. Load the filtered supernatant onto the HisTrap Column at 4 ml/min.
5. Run a 4 ml/min method on the ÄKTA system where after an initial 40 ml HisTrap Buffer A wash, a 40 ml 5 % HisTrap Buffer B wash is conducted. Finally, elute PTEN from the column using 40 ml of HisTrap Buffer B. It is advisable to then run an SDS-PAGE of the collected fractions to determine the location and purity of PTEN at this stage.
6. At this stage it is often preferable to remove the His-tag from PTEN. However, it may be desirable to retain the His-tag for certain experiments, in which case this cleavage step, and the subsequent second HisTrap purification can be skipped. Pool the PTEN-containing fractions, estimate the protein concentration by OD_{280}, and add TEV protease in a ratio of 1:20 TEV to PTEN (w/w).
7. Soak the SnakeSkin™ dialysis membrane in 4 l of dialysis buffer for 5 min. Pour the protein solution into the dialysis membrane, seal adequately with binder clips, and leave the protein solution overnight at 4 °C to digest and dialyze (use a stir bar to keep the solution moving). The purpose of this dialysis step is to remove the imidazole present in the PTEN solution, so that the solution can be passed back over a HisTrap column to remove the His-tagged TEV protease.
8. Repeat **step 5**, using the Dialysis Buffer in place of HisTrap Buffer A. Note well that the cleaved PTEN will now no longer bind to the HisTrap Column, and as such, it is imperative that the whilst loading the column the flow-through is collected and kept on ice. After running the HisTrap Column it is again advisable able to run an SDS-PAGE to determine that the protein is in the flow-through and that it has been correctly cleaved.

9. If necessary, an ion-exchange chromatography step can now be run to further purify PTEN prior to gel filtration. Equilibrate a HiTrap Q HP column with 20 ml Ion Exchange Buffer A run at 4 ml/min. Dilute the PTEN solution with an equal volume of Dilution Buffer before passing over the HiTrap Q HP column at 4 ml/min. Run a 4 ml/min method on the ÄKTA system where after an initial wash of 20 ml of Ion Exchange Buffer A, an increasing concentration of Ion Exchange Buffer B is run over 30 column volumes. It is strongly advised to run an SDS-PAGE to determine where PTEN has eluted from the HiTrap Q column. PTEN typically elutes at 200 mM NaCl (*see* **Note 3**).
10. Pool all PTEN containing fractions, and concentrate using a 10,000 MWCO Amicon Ultracentrifugation concentrator, run at 4 °C until a volume of approximately 1 ml is achieved. If required, PTEN can be dephosphorylated at this stage using lambda protein phosphatase (*see* **Note 4**) (*see* Subheading 3.2).
11. Inject the concentrated PTEN solution onto a Superdex 75 16/60 gel filtration column, equilibrated in gel filtration buffer, and run at 1 ml/min at 4 °C.
12. Pool and concentrate the PTEN containing fractions to at least 1 mg/ml for subsequent HDX-MS analysis. Aliquot, flash freeze in liquid nitrogen, and store at −80 °C.

3.2 PTEN Dephosphorylation and Re-phosphorylation

When purified from Sf9 cells, the C-terminus of PTEN is found primarily in a partially phosphorylated state (*see* **Note 4**). In order to investigate the structure of PTEN, it is necessary to first remove these modifications to produce a homogenous sample, and then re-phosphorylate with recombinant kinases in vitro if the phosphorylated state of the enzyme is desired.

1. Prior to the size exclusion chromatography step in the purification of PTEN, PTEN can be dephosphorylated. Incubate 1 ml of PTEN with 10 μl of Lambda protein phosphatase in a buffer (supplied by NEB) consisting of 50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01 % Brij 35, 1 mM $MnCl_2$, pH 7.5, for 90 min at 30 °C (*see* **Note 5**). The subsequent gel filtration step will then remove the lambda protein phosphatase and the required manganese.
2. In order to produce homogenously phosphorylated material, PTEN is subsequently re-phosphorylated in vitro using recombinant CK2 and GSK3β (both available from New England Biolabs). Following the gel filtration step (*after PTEN has been dephosphorylated using lambda protein phosphatase),* concentrate PTEN again, as previously described, to 500 μl total volume. Add 20 μl of 0.1 M ATP solution, 100 μl of the

GSK3β and CK2 reaction buffers supplied by New England Biolabs (*see* **Note 6**), and 5 μl each of the kinases GSK3β and CK2 solutions. Bring the final volume to 1 ml with the addition of 270 μl gel filtration buffer. The solution is then incubated at 25 °C for 24 h, and then the PTEN is purified using gel filtration as previously described.

3.3 Liposome Production

1. In a sterile, glass, screw-top vial mix the lipid stock solutions together in the desired ratio (*see* **Notes 7–9**). A plasma membrane mimicking composition [18] is the following: 5 % brain phosphatidylinositol 4,5-bisphosphate, 20 % brain phosphatidylserine, 45 % brain phosphatidylethanolamine, 15 % phosphatidylcholine, 10 % cholesterol, and 5 % sphingomyelin (all percentages given as weight/volume). A typical liposome stock preparation may be 1 ml of 5 mg/ml lipids.
2. Evaporate the organic solvents under a stream of nitrogen or argon gas, whilst turning the vial in your hands. Care should be taken that the stream of gas is not too powerful, as this may cause the lipids to be blown out of the vial. A smear of lipids can be observed on the side of glass vial, resembling spider's silk.
3. Place the vial in a vacuum desiccator (with the lid of the vial removed), and allow the organic solvents to be further evaporated at room temperature under vacuum for 1 h.
4. Remove the vial from the desiccator, and add 1 ml lipid buffer.
5. Place the lid back on to the vial, and further seal the lid with Parafilm®.
6. Vortex the vial for 3 min to resuspend the lipids.
7. Sonicate the vial in a bath sonicator for 2 min to remove any residual lipids still stuck to the glass.
8. Remove the lipids from glass vial and place in a 1.5 ml Eppendorf® tube.
9. Freeze the vial in liquid nitrogen until the lipid solution is opaque and frozen solid. Once frozen, rapidly remove the vial from the liquid nitrogen and place the vial in a 43 °C water bath until completely thawed. This is a single "freeze-thaw." Repeat this process a total of ten times (*see* **Note 10**).
10. Wash the Hamilton® Syringe sequentially in 70 % ethanol, water, and finally lipid buffer.
11. Aspirate the lipid solution into the syringe. Screw the membrane filter onto the end of the syringe until finger tight, and then pass the lipid solution through the filter, collecting the extruded lipid solution a suitable sterile container. Remove the filter from the syringe, and aspirate the solution once again.

Repeat this process until the lipids have been extruded a total of 11 times (*see* **Note 11**).

12. Aliquot the liposomes in a desired volume, freeze in liquid nitrogen and store at −80 °C. When using lipids in subsequent experiments, it is preferable to thaw them from −80 °C at room temperature.

3.4 HDX-MS Sample Preparation

In this experiment, a binding footprint for PTEN on a membrane bilayer is determined. The protein will be incubated with a D_2O containing buffer, either in the presence or absence of liposomes, at four different time points: 3, 30, 300, and 3000 s (*see* **Note 12**). This process can be automated using the LEAP Technologies Precise Adaptable Liquid-Handling system.

1. The composition of a single deuterated sample should be the following: 10 μl of 5 μM PTEN (dephosphorylated), 40 μl H_2O or D_2O solution, 20 μl quench solution (*see* **Note 13**). Each time point (of which there is four) is carried out in triplicate, in two conditions (with and without lipids), producing a total of 24 samples. In addition to this three non-deuterated samples are required for identification of PTEN's peptic peptides, bringing the total number of samples to 27.
2. Each sample will occupy a single 500 μl Eppendorf tube. It is necessary to label each tube with its contents (PTEN with/without liposomes), and time point, and also to clearly label the three non-deuterated samples.
3. Thaw the dephosphorylated PTEN on ice, and then remove any possible precipitated protein by using a spin filter (centrifuge at 13,000 × *g* for 10 min in a 4 °C). Measure the concentration of the flow-through. In this example, the concentration may be 40 μM.
4. Thaw the 5 mg/ml liposomes at room temperature (165 μl required).
5. Produce two 200 μl aliquots of two different protein solutions. Assuming a PTEN concentration of 40 μM, and a liposome stock concentration of 5 mg/ml:
   (a) PTEN Stock A (without liposomes): 25 μl of PTEN (final concentration 5 μM), 40 μl lipid buffer, 135 μl gel filtration buffer.
   (b) PTEN Stock B (with liposomes): 25 μl of PTEN, 40 μl of liposomes, 135 μl gel filtration buffer.
6. Aliquot 10 μl of the protein stocks into the 0.5 ml tubes and allow to equilibrate to room temperature for 10 min.
7. Produce 1 ml of the D_2O buffer solution. To 0.5 ml of this solution, add 125 μl of either 5 mg/ml liposomes or lipid buffer.

8. To each PTEN aliquot, add the appropriate D_2O Buffer solution, and mix thoroughly. Incubate PTEN with the D_2O with the appropriate timing, using a timer. It is critical that the timing is precise to about 1 s in order that replicates have similar rates of exchange. At the end of each time point, rapidly add 20 μl of the ice-cold quench solution, mix (using the pipette), close the Eppendorf tube, and freeze immediately in liquid nitrogen (*see* **Note 14**). For longer time points (300/3000 s) close the Eppendorf tube between adding the D_2O and quenching the reaction.
9. Prepare non-deuterated samples by diluting PTEN Stock A with the H_2O buffer and quenching (as no deuterium is being incorporated at this point the time of incubation is irrelevant).
10. Store samples at −80 °C until analysis.

3.5 Non-deuterated Samples: Identification of Peptides

In order to identify the peptides that are to be subsequently analyzed for perturbations in their rate of solvent accessibility, it is necessary to digest a non-deuterated PTEN sample, and run an MS/MS experiment (from which the sequences of the peptides can be determined). The mass spectrometer should be in positive ion mode and resolution mode, and calibrated (using, e.g., Glu-1-fibrinopeptide B) (*see* **Note 15**). Refer to Fig. 1 for a schematic of the fluidic system used, and how it is manipulated during the process.

1. Using the isocratic pump (with 0.1 % formic acid solution in Line A) equilibrate the immobilized pepsin cartridge with 0.1 % formic acid solution at a 0.3 ml/min flow rate for 10 min.
2. Using the dual-gradient analytical pump (with 0.1 % formic acid solution in Line A, and 100 % acetonitrile in Line B), equilibrate the C18 UPLC Column in 3 % acetonitrile (97 % 0.1 % formic acid) by flowing at 0.04 ml/min for 10 min. Ensure that the pressure on the system has stabilized, typically at 40 MPa bar for the dual-gradient pump, and 5 MPa for the isocratic pump.
3. Inject the PTEN sample onto the immobilized pepsin cartridge, and allow PTEN to be digested for 180 s.
4. Reverse the flow over the peptide trap, eluting the peptides from the trap and loading them onto the C18 Column.
5. Concurrently with **step 4**, start an increasing acetonitrile gradient, which over 24 min reaches 90 % acetonitrile.
6. Concurrently with **step 5**, begin collecting MS/MS data on the mass spectrometer.
7. Repeat the process after the data acquisition (**steps 1–6**), but collecting in MS mode.

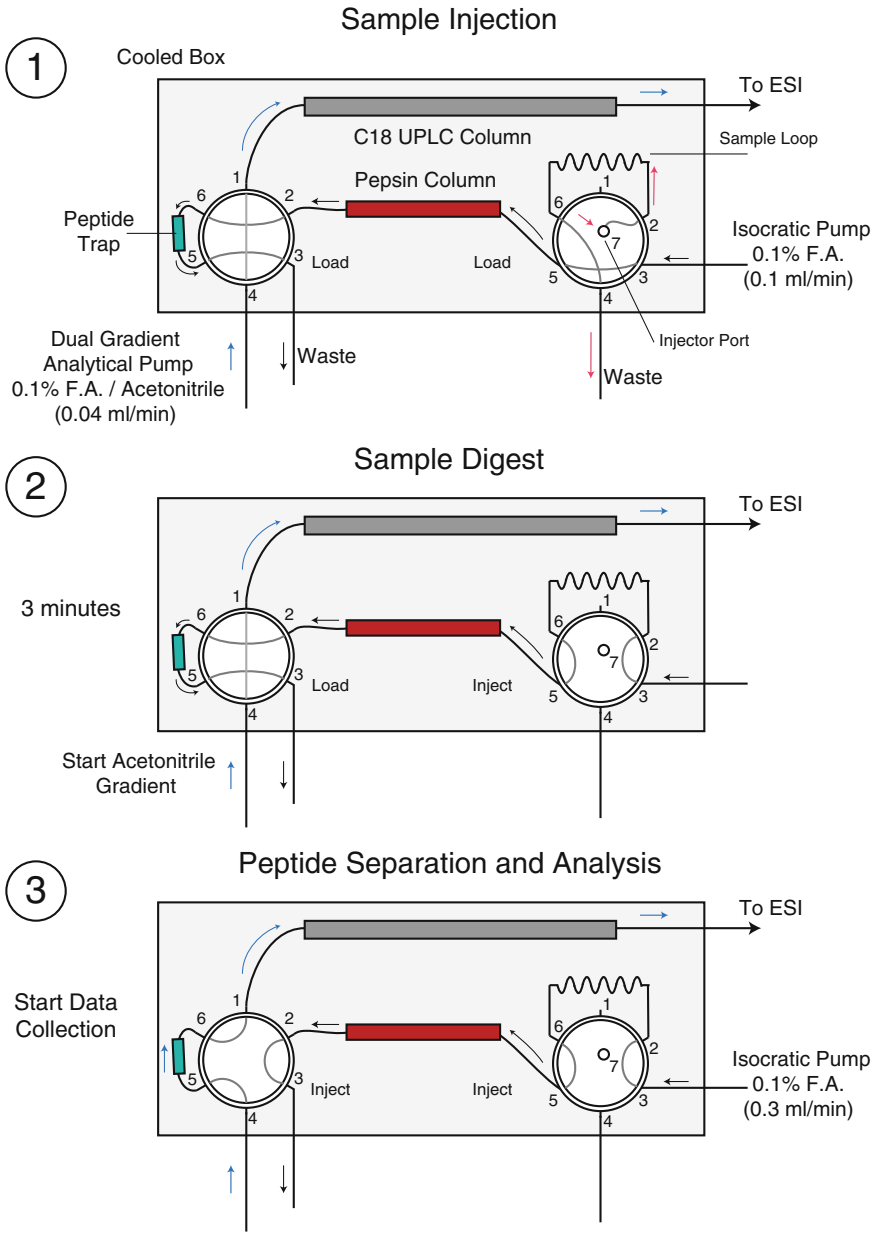

Fig. 1 Schematic of the UPLC system used in HDX-MS. (*1*) Both valves are in the "load" position. The 0.1 % formic acid solution from the isocratic pump flows over both the pepsin column and the peptide trap, eventually going to waste. The dual-gradient column pump flows onto the C18 UPLC column. The peptide sample is loaded at the injection point (labeled 7) onto the loop. (*2*) The right hand valve is switched to inject, emptying the contents onto the peptide trap. As the protein is digested, the peptides are immobilized onto the peptide trap. Digest the protein for 3 min. (*3*) The left hand valve is now switched to inject. The peptide trap is now in line with the acetonitrile gradient, but with the flow reversed. The peptide trap is also now in line with the C18 column. With the reversed flow, the peptides are eluted onto the column, and the acetonitrile gradient elutes the peptides from the C18 column to be subsequently analyzed using the mass spectrometer

3.6 Deuterated Samples: Identification of Peptides

The peptic digest and subsequent analysis of peptides of deuterated samples should be carried out as above, but in MS mode, rather than MS/MS mode. Care should be taken to ensure than the timing of the digestion and separation of peptides is carried out in a reproducible manner, as the deuterated sample is exposed to an aqueous environment, and back exchange will occur. For this reason, it is also imperative that the fluidics system maintains a low temperature (<4 °C).

3.7 Data Analysis

Currently we use HDExaminer (Sierra Analytics) for all data analysis, and further analyze the data produced with Microsoft Excel. Other software, such as DynamX (Waters) is available. The process of using your chosen software is beyond the remit of this chapter but can be briefly summated as follows:

1. Import the peptide list identified from the two MS/MS runs into the software (*see* **Note 16**).
2. Identify the non-deuterated peptides from the MS file. Manually check the peptides for correct selection and that each selected peptide is of suitable quality. This includes the peptide's retention time, charge state, and the presence of overlapping peptides that may influence the centroid mass calculation (*see* **Note 17**).
3. Add the files containing the data obtained from deuterated PTEN.
4. Ensure the deuterated peptides are of sufficient quality to produce reproducible data—repeating the quality assurance steps as mentioned for **Note 17** (*see* **Note 18**).
5. Compare the levels of deuterium incorporation for identical peptides in the two datasets to determine differences in HDX-rate (*see* **Note 19**).

4 Notes

1. Lipids can be ordered pre-dissolved in either a chloroform solvent or as a powdered stock, in which cause it is advisable to dissolve the powdered lipid in chloroform upon receipt and store at −80 °C. These materials are required to produce liposomes of the following composition: 5 % brain PIP_2, 20 % brain PS, 45 % brain PE, 15 % brain PC, 10 % cholesterol, 5 % sphingomyelin (all percentages given as weight/volume).
2. It is crucial to include EGTA in the lipid buffer to buffer Ca^{2+} in a systematic manner. This ion is vital for membrane fusion [19, 20], and in order to maintain reproducibility Ca^{2+} concentration must be controlled.

3. Two PTEN-containing peaks can often be observed when eluting from the HiTrap Q HP, one at 200 mM NaCl, and a second concentration of 400 mM NaCl or higher. This second peak is often misfolded protein, and will be present in the void of the gel filtration column.
4. If expressing PTEN with an intact C-terminus in a Eukaryotic expression system (i.e., containing residues 353–403) it is highly likely that the protein will be multiply and not homogeneously phosphorylated on the C-terminus [21, 22].
5. Mass spectrometry analysis found that there was no phosphorylated material after 90 min at 30 °C. The subsequent gel filtration step will then remove the lambda protein phosphatase (no phosphatase activity was determined in PTEN C124S that had been dephosphorylated using this method).
6. The NEB buffers contain the following (and are shipped as a 10× stock solution). CK2 reaction buffer: 200 mM Tris–HCl pH 7.5, 500 mM KCl, 100 mM $MgCl_2$. GSK3β reaction buffer: 200 mM Tris–HCl pH 7.5, 100 mM $MgCl_2$, 5 mM DTT. Therefore it is not necessary to add additional $MgCl_2$.
7. Pipetting organic solvents accurately can be challenging due to their low viscosity. Using a 200 μl pipette is preferable to a 1000 μl pipette as the larger bore size on the tip can lead to the solutions dribbling out.
8. Keep all lipid stocks dissolved in organic solvents sealed and on ice as much as possible. Owing to their volatility, the solvent will evaporate rapidly at room temperature, altering the concentrations of the stock solutions.
9. On addition of the PIP_2 to this lipid composition, the solution may turn cloudy, which is undesirable. Addition of 5 μl of methanol, and allowing the solution warm to room temperature will quickly reverse this.
10. Take note of the time required to freeze and thaw the lipids, and attempt to be as reproducible as possible for each freeze/thaw cycle. Do not, for instance, leave the lipids to thaw at 43 °C for longer than is absolutely necessary.
11. The unscrewing and rescrewing of the filter is difficult, and passing the lipid solution through the filter requires some effort to be applied to the syringe. Take care not to introduce air into the syringe, as bubbles can be formed in the liposome solution.
12. The "3 s" time point is difficult to conduct. Start by aspirating both the quench and the D_2O solution in the pipettes. Then add the D_2O Buffer, rapidly mix by aspirating up and down, and then add the quench, and flash freeze. Furthermore, by chilling the samples on ice, and also chilling the D_2O buffer

(and also preferably chilling the pipette tips and conducting the sample preparation step in a 4 °C room), a further time point of 3 s at 0 °C can be conducted. This is occasionally referred to as 0.3 s in the literature as there is an approximate tenfold decreased rate of solvent exchange between room temperature and 0 °C.

13. It is crucial to expose the two different set of samples (with and without lipids) to the exact same concentrations of D_2O, and also as far as possible, the exact same buffer, salt, and temperature conditions. Differing levels of D_2O between the samples will mask the perturbations of solvent exchange rate observed on membrane binding, and will prevent accurate data analysis.
14. Be sure to maintain sufficient distance between the liquid nitrogen container and the samples as to not chill them below room temperature during D_2O incubation.
15. If possible, an internal lock-spray calibration should also be run concurrently with the samples.
16. When exporting the identified peptides from the database, only export peptides that are well identified. This requires a ppm error that is commensurate with what would be expected from the instrument calibration, sufficient product ions to allow for confidence in the identification in the precursor ion, and an e-value that provides confidence that the identification is significant. The "ion score," an amalgam of these properties, can be used to this purpose. We use an ion score threshold of 15.
17. Peptides must meet certain criteria in order to ensure correct identification, and an accurate calculation of the centroid mass of the peptide. Ensure the following: that the charge state of the peptide (as seen within the software) matches the expected charge state; that there are no overlapping peptides—peptides that are also within the isotopic envelope of peptide of interest; that the intensity of the peptide is above background noise (typically about 5000 counts is the lower limit), and that the m/z shift is less than ±0.005.
18. The error between experiments, especially for the longer time points, should be certainly <3 %, and often within 1 %. The time point with the highest error associated is typically the 3 s time point, as this is carried out manually and has the greatest room for error. Peptides that are of poor quality, or incorrectly identified, will almost certainly have a higher error associated between the repeats. Hallmarks of incorrectly identified deuterated peptides are %D that are >100 %, large m/z shifts, and larger than 1-min deviations from the non-deuterated experiments, and negative values of deuteration.

19. We have two criteria for determining whether shifts in deuterium uptake are significant: the percentage difference (%D) and the absolute mass shift (Da). The %D is the percentage increase in mass that can be expected from a peptide (based on both the number of available imide groups, and the amount of deuterium in the buffer). A significant change in %D can be calculated with an unpaired *t*-test, and is typically on the order of 6 %. Obviously smaller peptides (e.g., 5 residues) that experience small mass differences will have exaggerated %D when compared to larger (10+ residue) peptides. To ensure confidence in the shifts seen in smaller peptides, we also require a mass difference of at least ±0.5 Da between the mean centroid mass value for both datasets.

Acknowledgement

This work was supported by the Medical Research Council (file reference U10518430).

References

1. Lee J.O. et al. (1999) Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. *Cell* **99**, 323–334
2. Kolmodin K., Åqvist, J. (2001) The catalytic mechanism of protein tyrosine phosphatases revisited. *FEBS Lett* **498**, 208–213
3. Xiao Y. et al. (2007) PTEN catalysis of phospholipid dephosphorylation reaction follows a two-step mechanism in which the conserved aspartate-92 does not function as the general acid — Mechanistic analysis of a familial Cowden disease-associated PTEN mutation. *Cell Signal* **19**, 1434–1445
4. Rodriguez-Escudero I. et al. (2011) A comprehensive functional analysis of PTEN mutations: implications in tumor- and autism-related syndromes. *Hum Mol Genet* **20**, 4132–4142
5. Zhang X.C., Piccini A., Myers M.P., Van Aelst L., Tonks N.K. (2012) Functional analysis of the protein phosphatase activity of PTEN. *Biochem J* **444**, 457–464
6. Lumb C.N., Sansom M.S.P. (2013) Defining the membrane-associated state of the PTEN tumor suppressor protein. *Biophys J* **104**, 613–621
7. Shenoy S. et al. (2012) Membrane association of the PTEN tumor suppressor: molecular details of the protein-membrane complex from SPR binding studies and neutron reflection. *PLoS One* 7, e32591
8. Rahdar M. et al. (2009) A phosphorylation-dependent intramolecular interaction regulates the membrane association and activity of the tumor suppressor PTEN. *Proc Natl Acad Sci USA* **106**, 480–485
9. Hopkins B.D. et al. (2013) A secreted PTEN phosphatase that enters cells to alter signaling and survival. *Science* **341**, 399–402
10. Pulido R. et al. (2014) A unified nomenclature and amino acid numbering for human PTEN. *Sci Signal* 7, pe15
11. Liang H. et al. (2014) PTENα, a PTEN isoform translated through alternative initiation, regulates mitochondrial function and energy metabolism. *Cell Metab* **19**, 836–848
12. Malaney P., Uversky V.N., Davé V. (2013) The PTEN Long N-tail is intrinsically disordered: increased viability for PTEN therapy. *Mol Biosyst* **9**, 2877–2888
13. Konermann L., Pan J., Liu Y-H. (2011) Hydrogen exchange mass spectrometry for studying protein structure and dynamics. *Chem Soc Rev* **40**, 1224
14. Burke J.E., Perisic O., Masson G.R., Vadas O., Williams R.L. (2012) Oncogenic mutations mimic and enhance dynamic events in the natural activation of phosphoinositide 3-kinase

p110α (PIK3CA). *Proc Natl Acad Sci U S A* **109**, 15259–15264

15. Vadas O. et al. (2013) Molecular determinants of PI3Kγ-mediated activation downstream of G-protein-coupled receptors (GPCRs). *Proc Natl Acad Sci U S A* **110**, 18862–18867
16. Burke J.E. et al. (2014) Structures of PI4KIIIβ complexes show simultaneous recruitment of Rab11 and its effectors. *Science* **344**, 1035–1038
17. Lee S.R. et al. (2002) Reversible inactivation of the tumor suppressor PTEN by H2O2. *J Biol Chem* **277**, 20336–20342
18. Vance J., Steenbergen R. (2005) Metabolism and functions of phosphatidylserine. *Prog Lipid Res* **44**, 207–234
19. McNew J.A. et al. (2000) Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature* **407**, 153–159
20. Nickel W. et al. (1999) Content mixing and membrane integrity during membrane fusion driven by pairing of isolated v-SNAREs and t-SNAREs. *Proc Natl Acad Sci U S A* **96**, 12571–12576
21. Miller S.J., Lou D.Y., Seldin D.C., Lane W.S., Neel B.G. (2002) Direct identification of PTEN phosphorylation sites. *FEBS Lett* **528**, 145–153
22. Torres J., Pulido R. (2000) The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus -implications for PTEN stability to proteasome-mediated degradation. *J Biol Chem* **276**, 993–998