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Peptide Pull-Down (PPD) Assay for Identification and Characterization of Histone PTM Effectors (PROT46)

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Introduction

Post-translational modifications (PTMs) of histones specify regulatory functions on chromatin through the recruitment of downstream effectors or "readers", that can specifically recognize different PTMs and translate epigenetic marks into a functionally relevant outcome (reviewed in Taverna *et al.*, 2007). To comprehend the complexity of epigenetic regulation, it is essential to not only catalogue histone PTMs and their patterns, but also to understand roles that histone PTMs and their effectors play in biological processes. An important component of this understanding will come through identification of histone PTM binding proteins. To this end, the peptide pull-down (PPD) assay provides a simple and effective tool to identify and characterize such reader proteins.

The general principle of the PPD is as follows. Biotinylated histone tail peptides containing a specific histone PTM and corresponding control unmodified peptides, are immobilized onto avidin-conjugated beads. The beads are incubated with a sample of interest, such as nuclear extract or purified recombinant protein, and washed to remove unbound proteins. Bound proteins can then be eluted and analyzed by SDS/PAGE and visualized by protein staining. By comparing proteins bound to modified versus unmodified peptides it is possible to identify candidate "reader" proteins for specific histone PTMs.

The PPD assay relies on two critical assumptions: 1) the "bait" peptides structurally mimic the histone region of interest, and 2) the length of the "bait" peptide is sufficient for recognition by the candidate reader protein(s). Histones are most heavily modified at their N- and C-terminal tails, which are relatively unstructured and jut away from the nucleosome/DNA core; thus short peptides are probably adequate structural-mimics. Also, structural studies of known histone PTM readers have revealed that generally fewer than ten residues of histone sequence are required for recognition (reviewed in Taverna *et al.*, 2007). However, there are still considerable limitations to the PPD assay. First, the recognition of some histone modifications (e.g., those within globular domains) may require structures that cannot be modeled by short, synthetic peptides. Second, the affinity of the reader of interest for its bait-peptide must be sufficiently high (low micromolar range) such that the interaction can withstand the washing conditions necessary to clear away non-specific binders. Third, the PPD assay is not quantitative.

Despite these limitations, the PPD assay has considerable advantages and applications. Namely, it enables an unbiased approach to identifying new epigenetic readers from a complex mixture of proteins. Second, it allows for the determination of substrate preference, specificity and recognition domains of candidate readers. Finally, it is a simple assay to perform, requires few reagents, and is relatively inexpensive.

Procedure

I. Design and synthesis of biotinylated "bait"-peptides:

Biotinylated histone peptides with specific modifications can be chemically synthesized or obtained commercially. Peptides should be HPLC purified to >80% purity, aliquoted, lyophilized, and stored dry at -80C. (See comment 1).

When designing peptides consider the following:

1. Peptides should be approximately 15-20 amino acids in length with the modification of interest in the center of the sequence and at least 6-8 flanking residues on each side. (See note 1)
2. Biotin should be conjugated to the N-termini of C-terminal histone peptides and the C-termini of N-terminal histone peptides
3. For a negative control: corresponding unmodified peptides should be synthesized for each modified residue
4. For a positive control: known histone PTM binding proteins can be used in conjunction with appropriately modified peptides (reviewed in Taverna *et al.*, 2007)

II. Preparation of peptide-bound resin:

Biotinylated peptides are conjugated to avidin beads to generate the resin used for the peptide pulldown. All steps should be performed either on ice or at 4°C unless noted otherwise. The following is written for a single peptide and should be performed in parallel for all peptides of interest.

1. Resuspend 100 µg of biotinylated peptide in 400 µL PBS. (See note 2)
2. Wash 400 µL avidin beads at least 3 times with 1 mL PBS + 0.1% Triton X-100, spin and remove supernatant. (See note 3)
3. Apply the 400 µL re-suspended peptide to the 400 µL washed beads
4. Incubate with rotation at room temperature for 3-5 hours to bind peptides to the beads. (See note 4)
5. Wash bound beads at least 3 times with 1 mL PBS + 0.1% Triton X-100 to remove unbound peptide
6. Resuspend peptide-conjugated beads in 400 µL PBS to yield a 50% slurry and store at 4°C. (See note 5) (See comment 2 and comment 3)

III. Peptide pull-down from a complex mixture of proteins:

The peptide pull-down (PPD) is an unbiased assay to identify novel "reader" proteins that bind to specifically modified peptides from a complex protein mixture such as a nuclear extract.

General notes for this procedure are as follows:

i. At each step of the PPD assay ensure inclusion of protease inhibitors to minimize protein degradation and phosphatase inhibitors if working with phosphorylated proteins/peptides
ii. All steps should be performed on ice or at 4°C unless noted otherwise
iii. As a negative control perform the PPD with un-conjugated avidin beads in addition to any peptide-conjugated beads being tested
iv. Other buffers besides HEPES can be used in this assay: PBS or Tris buffers at various salt concentrations have not had obvious detrimental effects in our PPDs
v. The following is written for a single peptide pull-down and should be performed in parallel for all peptides being tested

Prepare complex mixture of proteins:

1. Prepare fresh nuclear extract from 10^8 cells/pulldown using the cell type of choice and a standard high salt extraction protocol (Dignam *et al.*, 1983). (See note 6)
2. Adjust salt concentration of the extract to 150 mM and adjust the volume to 1 mL/10^8 cell equivalents (a total protein concentration of about 2-5 µg/µL) by either dialyzing against Buffer D(150 mM KCl) or diluting with Buffer D(no salt). (See note 7)
3. Add Triton X-100 to the extract to a final concentration of 0.1%. (See note 8)
4. Clear any precipitate from the extract by centrifuging in a microcentrifuge at max speed for 15-30

minutes and transferring the supernatant to a fresh tube. (See note 9)

Pre-clear complex mixture:

5. Aliquot 80 µL of 50% un-conjugated avidin bead slurry (40 µL of beads) and remove supernatant. (See note 10)
6. Wash avidin beads at least 1X in 500 µL Buffer D(150 mM KCl). (See note 11)
7. Add the prepared nuclear extract to the washed avidin beads and incubate at 4°C with rotation for 30 minutes. (See note 12)
8. Pellet beads by centrifuging in a microcentrifuge at 500 RCF for 30 seconds and collect the supernatant in a fresh tube to use for pull-down. (See note 13)
9. Remove 15 uL of the cleared extract and save it as the PPD "input" fraction for later analysis.

Prepare beads:

10. Aliquot 40 uL of 50% peptide-bound bead slurry (yields 20 uL of beads). (See note 14)
11. Pellet beads, remove all supernatant and ensure all aliquots have roughly equal amounts of beads. (See note 15)
12. Wash beads 1-3X in 500 uL Buffer D(150 mM KCl) and remove all supernatant. (See note 16)

Peptide pull-down:

13. Add the pre-cleared extract to the 20 uL of washed peptide-bound beads.
14. Incubate with rotation for 2-16 hours at 4°C to allow binding to peptide-bound beads. (See note 17)
15. Pellet beads by centrifuging in a microcentrifuge at 500 RCF for 30 seconds.
16. Transfer all of the supernatant to a fresh tube and save as the PPD "flow-through" fraction for later analysis.
17. Wash beads at least 5X in 1 mL Buffer D(300 mM KCl) and then remove and discard all supernatant. (See note 18)
18. Wash beads one last time with 1 mL Low-HEPES Buffer and remove and discard all supernatant. (See note 19)

Collect pull-down fractions:

19. Add 1-2 bead volumes (20-40 uL) of 100 mM glycine (pH 2.8) to the washed beads. (See note 20)
20. Incubate at room temperature for 10 minutes to acid-elute bound proteins. Flick tube gently during incubation to ensure efficient mixing of beads and elution buffer. (See note 21)
21. Transfer all of the eluate to a fresh tube with a capillary gel loading pipette tip.
22. Repeat steps 19-21 to perform a second acid-elution and pool both recovered eluates. (See note 22)
23. To neutralize the pH of the pooled eluates add 1/10 volume (4-8 uL) of 1 M Tris (pH 8.0).
24. Add the appropriate amount of 4X Laemmli sample buffer to the pooled eluates and save as the PPD "acid-elution" fraction for later analysis.
25. Resuspend acid-eluted beads in 1 bead volume (20uL) of 100 mM Tris (pH 8.0).
26. Add the appropriate amount of 4X Laemmli sample buffer and heat at 95°C for 5 minutes to denature any leftover bound protein. (See note 23)
27. Centrifuge in a microcentrifuge at max speed for 30 seconds, then vortex vigorously for 15 seconds and then centrifuge again.
28. Remove all supernatant as the PPD "denaturing-elution" fraction and save for analysis.

Analyze PPD fractions and identify bound proteins:

29. Analyze "input", "flow-through", "acid-elution" and "denaturing-elution" fractions by SDS-PAGE followed by silver staining using a mass spectrometry compatible protocol. (See note 24)
30. Excise gel bands present in the modified peptide, but not unmodified peptide, pull-down lanes ("acid-elution" and/or "denaturing-elution") and identify proteins by mass spectrometry.
31. Confirm the specific association of the identified protein with the modified peptide by analyzing the eluates by SDS-PAGE/immunoblotting with specific antibodies. Additionally, specific binding can be confirmed using recombinant protein in the PPD assay described below.

Alternative approaches for identification of proteins bound to specific peptides:

One major limitation of the peptide pull-down assay as described above is the relatively high background from non-specific binders which can obscure identification of specific readers of lower abundance. Another limitation is that the PPD is not quantitative. In an attempt to resolve these limitations two recently

reported proteomics approaches have coupled the PPD to stable isotope labeling by amino acids in cell culture (SILAC) or to a label-free spectral counting method (Vermeulen *et al.*, 2007; Chan *et al.*, 2009). In the SILAC-PPD approach extracts from cells grown in media containing stable isotope-labeled "heavy" amino acids are subjected to PPD with modified peptides, whereas extracts from cells grown in media with non-labeled "light" amino acids are pulled-down with unmodified peptides. The differentially labeled pull-down fractions are analyzed by mass spectrometry, where the ratio of heavy to light peptide for each protein can reveal relative recovery of that protein from the modified versus unmodified peptide baits. Notably, non-specifically binding proteins are easily identified as they have equal heavy to light peptide ratios. Alternatively, the spectral counting-PPD approach does not use labeled proteins and instead employs computer algorithms to estimate the relative abundance of an individual protein in modified versus unmodified peptide pull-downs from the spectral counts obtained in MS/MS analysis. Both approaches represent significant advances in overcoming the signal to noise and quantification limits of the PPD.

IV. Peptide pull-down using recombinant reader proteins:

Once a candidate reader protein has been identified purified recombinant protein can be used with the PPD assay to 1) determine whether the reader is directly binding to the modified peptide(s) of interest, 2) identify the region of the protein responsible for histone PTM recognition, 3) identify residues of the reader protein necessary for binding, or 4) characterize the specificity of the reader activity.

General notes for this procedure are as follows:

i. At each step of the PPD assay ensure inclusion of protease inhibitors to minimize protein degradation and phosphatase inhibitors if working with phosphorylated peptides
ii. All steps should be performed on ice or at 4°C unless noted otherwise
iii. As a negative control always perform the PPD with un-conjugated avidin beads in addition to any peptide-conjugated beads being tested.
iv. Other buffers besides PBS can be used in this assay: HEPES or Tris buffers at various salt concentrations have not had obvious detrimental effects in our PPDs. Thus, we recommend using a buffer that is most compatible with the recombinant protein of interest
v. When using a reader protein with a GST-tag, we strongly recommend proteolytically-cleaving off the tag. GST-domains on fusion proteins dimerize strongly, which can have a profound effect on the apparent affinity of the reader protein for the target peptides. (Maru *et al.*, 1996)
vi. The following is written for a single peptide pull-down and should be performed in parallel for all peptides being tested

Prepare protein sample:

1. Dilute 1-10 ug of purified recombinant protein into 400 uL Dilution Buffer. (See note 25)
2. Add 25 ug of BSA to the ~400 uL diluted protein solution. (See note 26)
3. Clear any precipitated protein from the solution by centrifuging in a microcentrifuge at max speed for 15-30 minutes and transferring the supernatant to a fresh tube. (See note 9)
4. Remove 15 uL of the cleared supernatant and save it as the PPD "input" fraction for later analysis.

Prepare beads:

5. Aliquot 30 uL of 50% peptide-bound bead slurry (yields 15 uL of beads). (See note 27)
6. Pellet beads, remove all supernatant and ensure all aliquots have roughly equal amounts of beads. (See note 15)
7. Wash beads 1-3X in 500 uL Wash Buffer and remove all supernatant. (See note 16)

Peptide pull-down:

8. Add the 400 uL of diluted/cleared protein solution to the 15 uL of washed peptide-bound beads and mix by gently flicking the tube.
9. Incubate with rotation for 1-4 hours at 4°C to allow binding of reader protein to peptide. (See note 17)
10. Pellet beads by centrifuging in a microcentrifuge at 500 RCF for 30 seconds.
11. Transfer all of the supernatant to a fresh tube and save as the PPD "flow-through" fraction for later analysis.

12. Wash beads at least 3X in 500 uL Wash Buffer and then remove and discard all supernatant. (See note 18)

Collect pull-down fraction and analyze:

13. Resuspend washed beads in 40 uL 1X Laemmli buffer (4X Laemmli sample buffer diluted 1:4 into Wash Buffer) and boil at 95°C for 5 minutes to denature the bound reader protein.
14. Centrifuge in a microcentrifuge at max speed for 30 seconds, then vortex vigorously for 15 seconds and then centrifuge again.
15. Remove all supernatant as the PPD "pull-down" fraction and save for analysis.
16. Analyze "input", "flow-through" and "pull-down" fractions by SDS-PAGE and Coomasie BB staining and/or SDS-PAGE coupled to immunoblot analysis with specific antibodies.

Materials & Reagents

Preparation of peptide-bound resin:

Biotinylated histone peptides (see procedure section)
Avidin agarose (Pierce)
Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4
Triton X-100 (20% v/v stock solution)
Sodium azide (10% w/v stock solution)

Peptide pull-down from a complex mixture of proteins:

Complex protein mixture (e.g., nuclear extract)
Buffer D: 20 mM HEPES, pH 7.9, 20% v/v glycerol, 0.2 mM EDTA, 0.2% Triton X-100, 2 mM dithiothreitol (DTT), 0.2 mM PMSF, protease inhibitor cocktail (Complete – Roche), KCl at the indicated concentration
Phenylmethanesulphonylfluoride (PMSF; 100 mM stock solution in ethanol)
Triton X-100 (20% v/v stock solution)
Avidin agarose (Pierce)
Peptide-bound resin (50% slurry; see procedure section)
Low-HEPES Buffer: 4 mM HEPES, pH 7.9, 10 mM NaCl
100 mM Glycine (pH 2.8)
Optional (for base-elution): 0.5 N NH_4OH + 0.5 mM EDTA
Optional (for peptide-elution): 0.5 mg/mL non-biotinylated peptide in PBS
1 M Tris (pH 8.0)
4X Laemmli sample buffer: 0.2 M Tris–HCl, pH 6.8, 8% SDS, 40% glycerol, 0.04% bromophenol blue, freshly added DTT to 40 mM
100 mM Tris (pH 8.0)
Silver staining kit (SilverQuest - Invitogen)

Peptide pull-down using recombinant reader protein:

Dilution Buffer: PBS, 0.1% Triton X-100, 2 mM dithiothreitol (DTT), 0.2 mM PMSF, protease inhibitor cocktail (Complete – Roche)
Phenylmethanesulphonylfluoride (PMSF; 100 mM stock solution in ethanol)
Purified recombinant reader protein (1-10 µg per pull-down)
Bovine Serum Albumin (BSA; 10-50 µg per pull-down)
Peptide-bound resin (50% slurry; see procedure section)
Wash Buffer: PBS, 150 mM NaCl (final salt concentration at 300 mM), 0.1% Triton X-100, 2 mM dithiothreitol (DTT), protease inhibitor cocktail (Complete – Roche)
4X Laemmli sample buffer: 0.2 M Tris–HCl, pH 6.8, 8% SDS, 40% glycerol, 0.04% bromophenol blue, freshly added DTT to 40 mM

Author Notes

1. For modified residues close to the ends of histone tails centering the modification is not possible, but otherwise centering is recommended.

2. This amount of peptide is sufficient to prepare 400 µL peptide-conjugated resin, which is enough for approximately 20 pull-downs. If resin with a different biotin-binding capacity is used scale the peptide:avidin ratio appropriately.
3. Use 400 µL of avidin beads per peptide, plus prepare an additional 400 µL to be used as an avidin only control. For washing: spin down beads in a microcentrifuge at 500 RCF for 30 seconds and remove supernatant with a capillary gel loading pipette tip to avoid removing beads.
4. Alternatively, one can incubate the beads with peptide overnight at 4°C
5. For long term storage of resin add sodium azide to a final concentration of 0.1%. Peptide bound beads can be stored stably at 4°C for at least a month. Methylated peptides are usually very stable, whereas phosphorylated peptides are more susceptible to hydrolysis.
6. The amount of necessary input material will vary depending on reader protein abundance, but 10^8 cells are usually sufficient. It is best to use freshly prepared nuclear extract to limit protein degradation, but extract can be flash-frozen in liquid nitrogen and stored at -80°C. It is important to try extracts from various cell lines because some factors may not be expressed well in a given cell type. Additionally, consider the cellular process in which the histone modification of interest is involved: for a PTM associated with mitosis, synchronized mitotic extracts would be the appropriate input. Some histone-bound factors will not efficiently extract with a 420mM salt extraction procedure (e.g. ones that bind chromatin tightly), in which case an alternative extraction procedure, such as solubilizing the chromatin by treatment with deoxyribonuclease (DNase) or micrococcal nuclease (MNase), could be advantageous.
7. It is important to use Buffer D with freshly added DTT, PMSF and protease inhibitors
8. The addition of Triton X-100 detergent minimizes non-specific binding of protein to peptide during the pull-down.
9. We centrifuge at 13200 RPM in a table-top microcentrifuge set at 4°C and adjust the time to ensure all visible precipitate is cleared from the extract. It is important to remove all precipitate, as it significantly increases the nonspecific background in the assay.
10. Centrifuge at 500 RCF for 30 seconds in a microcentrifuge to pellet the beads and then remove all supernatant with a capillary gel loading tip.
11. For washing: spin down beads in a microcentrifuge at 500 RCF for 30 seconds and remove supernatant with a capillary gel loading tip in order to avoid removing beads. One wash is usually sufficient to prepare beads for pre-clearing.
12. Incubation time can be increased if convenient, but we did not observe any further improvement in the nonspecific background reduction with pre-clearing times longer than 30 minutes. Also, please note that this step will not quantitatively remove non-specific binders from the extract.
13. If necessary, spin again to remove any residual beads.
14. The amount of peptide-bound beads can be adjusted depending on the abundance and binding affinity of the reader protein, but we have found that increasing the bead volume over 20 uL usually does not improve specific binder recovery, but can increase background levels.
15. Pellet beads by centrifuging for 30 sec at 500 RCF in a microcentrifuge and remove supernatant with a capillary gel loading pipette tip. It is important to ensure that roughly the same amount of peptide-bound beads is used for each pull-down to allow for comparisons among peptides.
16. For washing: spin down beads in a microcentrifuge at 500 RCF for 30 seconds and remove supernatant with a capillary gel loading pipette tip. After the last wash it is vital to remove as much supernatant as possible from beads.
17. Pull-down incubation time can be adjusted depending on the amount of protein/peptide used and the binding affinity. Overnight pull-downs can be performed for convenience, but 2 hours is generally sufficient for binding to occur and in some cases longer incubation times decrease the recovery of specific readers.
18. Thorough washing and removal of all supernatant from the bead pellet is essential to ensure no unbound protein is recovered in the pull-down fraction. The key to a successful PPD is a balance between stringent enough washing to remove background associations but mild enough washing to

maintain relevant associations. Optimal washing conditions vary between proteins and thus we recommend trying a variety of salt (150-500 mM) and detergent (0.1-0.5 %) concentrations.

19. Using Low-HEPES Buffer allows for changing pH readily during elution with glycine in the next step.
20. Adjust the volume for elution depending on desired recovery volume. Not all proteins will efficiently acid-elute with glycine, in which case there are two alternatives. First alternative: base-elute with 0.5 N NH_4OH + 0.5 mM EDTA. Second alternative: elute with 0.5 mg/mL non-biotinylated peptide in PBS (this is the most specific elution approach, but it is often inefficient and requires a large amount of peptide).
21. Incubation time should be increased if peptide elution is used instead of acid or base elution.
22. A second elution improves recovery of the bound protein from beads, but can be omitted to limit the eluate volume.
23. Collecting the remaining bead-bound protein by denaturation after acid-elution allows for the parallel comparisons between proteins that acid-eluted and those that remained bound to beads. This is important given that some proteins do not efficiently acid-elute.
24. We use the SilverQuest silver staining kit from Invitrogen. Handle the gel with care to avoid contamination with keratin.
25. It is important to use Dilution Buffer with freshly added DTT, PMSF and protease inhibitors. The amount of purified recombinant protein can be adjusted from 1-10 ug depending on binding affinity to the modified peptides (we start with 3 ug and adjust as needed). To minimize precipitation when diluting protein we recommend pipetting slowly while swirling pipette tip.
26. BSA is added to the protein input as an internal control and should not be recovered in the pull-down fraction. The amount of BSA can be varied from 10-50 ug as desired, but we use 25 ug to start.
27. The amount of peptide-bound beads can be adjusted depending on the binding affinity of the reader protein, but we have found 15 uL to be sufficient to visualize input proteins by Coomasie BB staining.

Reviewer Comments

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1. We aliquot peptides in concentrations of 1 mg/ml in H_2O, lyophilize and store peptide powder at -80°C (1 mg or 0.5 mg aliquots). One problem that we have encountered is that some peptides, because of their amino acid composition, are not soluble in water. An alternative approach resolving this problem is to resuspend these peptides (also the corresponding control peptides) in DMSO. We have used 16 µl DMSO for 500 µg peptides. Aliquot and store the solution at -80°C. Note that this solution does not freeze. When using these peptides in PPD assays, dilute to a final concentration of 0.1% - 1% DMSO.
2. It is also possible to incubate less peptides with less beads just prior to the PPD assay. This way, the coupled peptide beads are fresh and no sodium azide needs to be added for storage.
3. Instead of agarose-avidin beads it is possible to use magnetic beads coupled with streptavidin (Invitrogen; Dynal). These beads are more expensive, but are easier to handle (magnet instead of centrifugation). We had very good results using these beads in a PPD assay with a western blot detection of our protein of interest at the end.

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