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Mass Spectrometry of Protein Post-Translational Modifications Protocol

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Abstract

This protocol describes harvesting whole-cell protein lysates in RIPA buffer, immunoprecipitating a target protein (e.g., FOXA1) using a specific antibody with Protein A magnetic beads alongside an IgG control, and validating target enrichment by Western blot using input, IP, flowthrough, and control fractions. Enriched IP material is then resolved by SDS-PAGE, Coomassie-stained, excised around the target molecular weight, and prepared for LC-MS/MS to identify peptides and post-translational modifications, with database searching and FDR-controlled peptide filtering.

Expected results include robust enrichment of the target protein in the specific IP compared with IgG and flowthrough, confirming successful pulldown prior to mass spectrometry. LC-MS/MS analysis should yield high-confidence identification of target-derived peptides and associated PTMs (e.g., phosphorylation, acetylation, ubiquitinylation), with low false-discovery rates and minimal background contributions from gel-only controls.

Materials

1. 10 cm cell culture dishes
2. Phosphate-buffered saline (PBS), ice-cold
3. RIPA lysis buffer
4. 5 mL tubes
5. Target protein primary antibody (example: FoxA1/HNF3 α , clone E7E8W) — Cell Signaling Technology — Cat #53528
6. Isotype control antibody (example: Normal Rabbit IgG) — Cell Signaling Technology — Cat #2729 / 2729S
7. Protein A magnetic beads (Dynabeads Protein A for IP) — Thermo Fisher Scientific — Cat #10002D
8. Magnetic rack/stand for bead separation
9. Tube rotator (4 °C incubation)
10. Protease inhibitor cocktail
11. Phosphatase inhibitor cocktail
12. Laemmli sample buffer (2X)
13. β -mercaptoethanol
14. Pre-cast SDS-PAGE gel for Western blot (4–20% gradient, 15-well, 15 μ L) — Bio-Rad — Cat #4561096
15. Gel electrophoresis tank + power supply
16. Nitrocellulose membrane — Bio-Rad — Cat #1704270
17. Protein transfer system — Bio-Rad — Cat #1704150
18. TBS-T (Tris-buffered saline + Tween)
19. Nonfat dry milk (for 5% milk-TBST block)
20. Loading control primary antibody (example: anti-Vinculin)
21. Species-appropriate secondary antibody(ies)
22. Western blot imaging system (chemiluminescent or equivalent)
23. Pre-cast gel for MS sample separation (7.5%, 10-well) — Bio-Rad — Cat #4561024
24. Milli-Q (or equivalent) ultrapure water
25. Methanol (for gel fixing/destaining)
26. Acetic acid (for gel fixing/destaining)
27. Coomassie Brilliant Blue R-250 staining solution — Bio-Rad — Cat #1610436
28. Acetonitrile (for washing gel pieces)
29. Clean scalpel/razor blades and gel-cutting surface
30. –20 °C freezer (storage of gel pieces)
31. LC–MS/MS system (microcapillary reversed-phase LC coupled to tandem MS)
32. Trypsin (for peptide digestion; typical for LC–MS/MS)
33. Mascot software v2.6 — Matrix Science
34. Scaffold Q+S v5.0 — Proteome Software, Inc.



Troubleshooting

Problem

Low coverage of target protein, or lack of peptides for covered regions

Solution

Low coverage of target protein, or lack of peptides for covered regions can most easily be overcome by the use of larger amounts of initial protein (and by extension pulldown antibody and magnetic beads).

Sample Preparation

- 1 Conduct protein harvest of cells on three 10cm Dishes for each condition, by washing cells in ice-cold PBS, scraping in 1000 μ L/dish of RIPA lysis buffer per dish, transferring to individual 5mL tubes, incubating at 4°C for 30 minutes, and freezing at -80°C.

Immunoprecipitation of Target Protein

- 2 Conduct immunoprecipitation of samples harvested in RIPA, after first spinning down at max G and removing cell debris pellet.
 - Input samples were prepared by taking 100 μ L from each sample, and were stored at -20°C.
 - IP samples were prepared by taking 2.1mL of each sample, and incubating with target protein antibody (eg: α -FoxA1, 5 μ g, Cell Signaling, #53528, clone E7E8W) according to manufacturer's instructions, for 2 hours at 4°C on a rotator.
 - IgG control samples were prepared by taking 410 μ L of each sample and incubating matched IG control antibody (eg: Rabbit IgG, 1 μ g, Cell signaling, #2729S) according to manufacturer's instructions, for 2 hours at 4°C on a rotator.
- 2.1 Conduct binding of antibody-target complexes from IP and IgG control samples by incubating with 50-250 μ L of Protein-A DynaBeads (Thermo Scientific, #10002D) each according to manufacturer's instructions, overnight at 4°C on a rotator.
- 2.2 Conduct pulldown of protein lysates incubated with α -FoxA1 antibody (or other target), IgG control and Dynabeads overnight.
 1. Magnetic bead pellets from FoxA1 pulldowns are separated from supernatant, and all but 100 μ L the supernatant was discarded for Flowthrough analysis.
 2. Magnetic bead pellets from FoxA1 pulldowns are washed five times with 400 μ L of RIPA plus protease and phosphatase inhibitors.
 3. Re-suspend magnetic bead pellets in 100 μ L of RIPA, and transfer to a clean tube (to avoid co-elution of proteins bound to the tube wall).
 4. Magnetic bead pellets from FoxA1 pulldowns are separated from supernatant, and the supernatant was replaced with 65 μ L of RIPA + 2X Laemmli buffer for FOXA1 IP's and 20 μ L of RIPA + 2X Laemmli buffer for IgG IP's.
 5. Samples were heated at 70°C for 10 minutes to elute bound proteins.
 6. Magnetic bead pellets were separated from supernatant, and the supernatant (containing the FoxA1 proteins) are transferred to a new clean tube where 2.5% β -mercaptoethanol was added.



Western Blot Validation

- 3 Conducted Western blot of Input, IgG, FOXA1 IP and IP Flowthrough samples (prepared in Laemmli buffer and 2.5% β -mercaptoethanol) and run gel electrophoresis, as below.



- 3.1 Load Input, IgG, FOXA1 IP and IP Flowthrough samples onto the same BioRad pre-cast 4-20% 15-Well gradient gel.
 - NOTE: Place an empty well between sample sets to ensure no overflow contaminates IgG for example.
- 3.2 Run gel at 10 milliamps constant for 30 minutes (to ensure the entire sample enters the gel at the same time), and 50 milliamps constant for the remaining time (until dye front reaches appropriate distance or runs off).
 - NOTE: Do not run the gel so long as to allow your protein of interest to exit the gel.
- 3.3 Conduct fast-transfer of gel to Nitrocellulose membrane (BioRad, #1704270) at 2.5 amps and 25 volts for 7 minutes in a Trans-Blot Turbo Transfer System (BioRad, #1704150), and block in 5% MILK-TBST for 60 minutes.
- 3.4 Block membrane in 5% MILK-TBST for 60 minutes.
- 3.5 Washed membranes twice in TBS-T, and incubated with a suspension of target (eg: α -FOXA1), and a suspension of loading control (eg: α -Vinculin) antibodies, overnight at 4°C on a rocker. 
- 3.6 Wash membranes three times in TBS-T for 15 minutes total, and incubate with a suspension of appropriate secondary antibody based on target and loading control primary antibody hosts, at room temperature for one hour on a rocker. 
- 3.7 Wash membranes three times in TBS-T for 15 minutes total, and visualize membranes to confirm enrichment of target (eg: FOXA1) in IP samples compared to Input, IgG and flowthrough samples. 

Mass Spectrometry Sample Preparation

- 4 Conducted gel electrophoresis of FOXA1 IP samples (prepared in Laemmli buffer and 2.5% β -mercaptoethanol) and run, as below.
- 4.1 Load IgG and FOXA1 IP samples onto the same BioRad pre-cast 7.5% 10-Well gradient gel.
 - NOTE: Place an empty well between sample sets to prevent capturing adjacent samples when cutting the gel below.
- 4.2 Run gel at 10 milliamps constant for 30 minutes (to ensure the entire sample enters the gel at the same time), and 50 milliamps constant for the remaining time (until dye front reaches appropriate distance or runs off).
 - NOTE: Do not run the gel so long as to allow your protein of interest to exit the gel.



- 4.3 Wash the MassSpec sample gel three times for 5 min with clean Milli-Q H₂O, and then fixed with 40% methanol, 10% acetic acid, and 50% H₂O for 1 hour.
- 4.4 Wash the MassSpec sample gel three times for 5 min with clean Milli-Q H₂O, and then stain with Coomassie Blue solution (BioRad, #1610436) for 45 minutes with gentle shaking.
- 5 Wash the MassSpec sample gel three times for 5 min with clean Milli-Q H₂O, and destain with 40% methanol, 10% acetic acid, and 50% H₂O overnight with gentle shaking.
- 5.1 Wash the MassSpec sample gel three times for 5 min with clean Milli-Q H₂O, imaged, and visible bands cut out around the 50kDa marker for FOXA1, and one empty lane cut out for Gel Background sample.
- 5.2 Wash cut bands twice in 50% acetonitrile, and store in a -20°C freezer until ready for Mass Spectrometry analysis of post-translational modifications of target protein (eg: FOXA1).



Digestion and Mass Spectrometry Analysis

- 6 Based on protein of interest, peptides are typically trypsinized, separated and analyzed by microcapillary reversed-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to manufacturer's instructions.
- 6.1 Based on LC-MS/MS equipment and software available, MS/MS spectra are typically analyzed using Mascot version 2.6 (Matrix Science) by searching the reversed and concatenated human protein database (<http://www.ebi.ac.uk/uniprot/database/download.html>) with a parent ion tolerance of 15 ppm and fragment ion tolerance of 0.05 Da.
 - Carbamidomethylation of Cys are specified as a fixed modification and oxidation of Met, deamidation of Asp/Gln, phosphorylation of Ser/Thr/Tyr, ubiquitinylation of Lys, methylation and dimethylation of Lys/Arg and acetylation of Lys/Arg as variable modifications.
 - Results are imported into Scaffold Q + S 5.0 software (Proteome Software, Inc.) with a peptide false discovery rate (FDR) of ~1.1%.