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Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) Cofractionation and In-Gel Digestion

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We use this protocol and it's working

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Abstract

This protocol is used to isolate protein complexes by blue-native page, a type of native gel. The source of proteins for separation in this example is early endosomes isolated by Endo-IP (with Flag-tagged EEA1 as an affinity handle).

Materials

n-Dodecyl β -D-maltoside (DDM, Gold Biotechnologies, DDM5)

NativeMark Protein Standard (Invitrogen, LC0725)

NativePAGE 4-6% Gels (Invitrogen, BN1002BOX)

tris(2-carboxyethyl)phosphine (TCEP; Gold Biotechnology, 51805-45-9)

2-chloroacetamide (Sigma-Aldrich, C0267)

trypsin (Promega, V511C)

Lys-C (Wako Chemicals, 129-02541)

KPBS buffer (25 mM KCl, 100 mM potassium phosphate, 150 mM NaCl, pH 7.2)

MultiScreen Filter Plates (Sigma Millipore, MSHVN4510)

Troubleshooting



Sample Preparation

- 1 Generate the sample for analysis, such as a purified organelle (Endo-IP in this case). The relevant protocol for Endo-IP can be found at:
<https://doi.org/10.17504/protocols.io.ewov14pjyvr2/v2>

Separation on BN-PAGE

- 2 Resuspend freshly prepared purified endosomal pellets in 40 μ L of KPBS containing 0.5% n-Dodecyl β -D-maltoside (DDM).
- 3 Extract proteins by rotating the mixture for 45 minutes at 4°C.
- 4 Clarify the protein extracts by centrifugation at 20,000 x g for 20 minutes at 4°C.
- 5 Mix the clarified extracts with 10 μ L of BN loading buffer, 1 μ L of Coomassie G-250 mix, and 0.5 μ L of a native molecular weight marker.
- 6 Load the samples onto a 4–16% NativePAGE gel.
- 7 Run the gel at 150 V for 1.5 hours, followed by 250 V for 20 minutes at 4°C.
- 8 Fix the gel in 50% ethanol and 3% phosphoric acid. Reconstitute in water.
- 9 Stain the fixed gel with Coomassie G-250 dye.

In-gel digestion

- 10 Slice each sample lane from the gel into 48 1-mm slices.



- 11 Transfer the gel slices to a 96-well filter plate for in-gel digestion.
- 12 Reduce the proteins by adding 100 μ L of 5 mM tris(2-carboxyethyl)phosphine (TCEP) in 50 mM ammonium bicarbonate and incubate for 30 minutes at 37°C.
- 13 Alkylate the proteins with 20 mM chloroacetamide in 50 mM ammonium bicarbonate for 15 minutes at room temperature.
- 14 Destain with 50% acetonitrile in 50 mM ammonium bicarbonate, and dry in 100% acetonitrile.
- 15 Digest proteins with 0.2 μ g Lys-C for 4 hours at 37°C, followed by an overnight incubation with 0.2 μ g trypsin.
- 16 Extract the resulting peptides from the gel by increasing concentration of acetonitrile up to 70%.
- 17 Dry the peptide extracts using a SpeedVac concentrator. Reconstitute the dried peptides in a solution containing 5% acetonitrile and 5% formic acid.
- 18 Samples are now ready for liquid chromatography-tandem mass spectrometry (LC-MS/MS).