iBioID Proteomics Protocol

Soderling Lab: Please reference *Science* **353**, 1123–1129 (2016)
Last updated: July 28th, 2016

Notes: Many of the below procedures should be done under "keratin free" conditions. This means use only powder-free Nitrile gloves (NOT Latex) in a laminar flow hood.

Specialized Reagents to order (name: company, catalog #)
Biotin: Sigma; B4501
Ammonium Bicarbonate (Ambic): Sigma; A6141
Deoxycholate: Fisher; BP349-100
Protease Inhibitors: RPI; (AEBSF-A20010)(Leupeptin-L22035)(PepstatinA-P30100)
HA antibody resin: Sigma; A2095
Low protein binding 1.5ml eppendorf tubes: Sigma; Z666505
NeutrAvidin: Thermo; 29201
Slide-A-Lyzer MINI Dialysis 3,500 MWCO: Thermo; 69550
RapiGest SF Surfactant: Waters; 186001861
Spin Cups-Cellulose Acetate Filter: Thermo; 69702
pyruvate carboxylase Ab: Invitrogen; A21970
MAP2 Ab: GeneTex; GTX49163
Protein G agarose (capacity 20mg IgG/mL): Millipore; 16-266
Mass spec compatible water: Honeywell; LC365-1
BCA protein quantification kit: Thermo; 23225

Equipment needed:
Micro Tissue Grinder or dounce homogenizer (2ml)
Sonicator
Ultracentrifuge

Solutions:

- Make 1M Ammonium Bicarbonate (Ambic) in keratin free condition.
- Make 2%SDS, 10mL in keratin free condition.
- Make 1M NaCl, 10mL in keratin free condition.
- Make 1% TritonX100, 1% deoxycholate, 25mM LiCl, 10mL in keratin free condition.
- Make 50mM Ambic in keratin free condition.
- Make 0.1% RapiGest / 1mM biotin / 50mM Ambic (200uL of 5mM biotin/50mM Ambic solution, 40uL of 1M Ambic, 760uL of dw in 1 vial RapiGest) in keratin free condition.
- Make 50mM Ambic, 500mL x 2. Keep the beaker in the cold room a day before the experiment.
  - [Ambic FW=79.06, to make 50mM 1.976g/500mL]

- Lysis(-RIPA) buffer [50mM Tirs/Cl pH7.5, 150mM NaCl, 1mM EDTA]

- 2x RIPA buffer [50mM Tirs/Cl pH7.5, 150mM NaCl, 1mM EDTA, 0.4%SDS, 2%TritonX100, 2% deoxycolate]

Day 1

Preparations:

1) Per sample: Pre-wash 20uL NeutrAvidin slurry with 2xRIPA buffer, 5 times
2) Per sample: Incubate 20uL of pyruvate carboxylase Ab with 10uL of Protein G agarose in 150uL of 0.1% TritonX-100/PBS + PIs, o/n at 4 degrees with rocking.
3) Per sample: Incubate 20uL of MAP2 Ab with 10uL of Protein G agarose in 150uL of 0.1% TritonX-100/PBS + PIs, overnight at 4* with rocking.

Procedures:

1) Set aside 3 eppendorf tubes (1.7 mL) per sample
2) Take tissue samples from biotin labeled mice from liquid nitrogen and keep on dry ice
3) Homogenize sample with 300 uL of Lysis-R buffer (including protease inhibitors). This should be done using 15 strokes with a glass dounce homogenizer (2ml, Pierce).
4) Transfer to an eppendorf tube, add 350 uL of 2x RIPA buffer and spin in microfuge at 15,000xg for 10 min at 4*C.
5) Sonicate sample at level 8, 7 sec.; Cool on ice for 10 min, Repeat Sonication and ice for a total of 3x.
6) Centrifuge at 15,000 rpm for 10 min, Transfer the supernatant to a new eppendorf tube.
7) Centrifuge in the ultracentrifuge (TLA-55, 40,000 rpm [100,000xg], 30 min, 4*C).
8) Transfer supernatant to a new eppendorf tube and add SDS to a final 1% concentration.
9) Briefly vortex and spin @ RT in microcentrifuge at 15,000xg for 10min.
10) Boil (95*C for 5 min), Cool on ice
11) Microcentrifuge at 15,000xg for 30 min at 4*C
12) Transfer supernant to a 15 mL conical tube and add protease inhibitors
13) Incubate with 17 uL of washed NeutrAvidin beads overnight at 4* with rocking.

Day 2

Preparations:

1) Pre wash the HA beads (100uL beads) in keratin free condition in low binding tube. Wash with 1x RIPA buffer first and wash with 50mM Ambic x 5 in keratin free condition.
2) Pre wash the spin cups (your samples x2) in 50mM Ambic x 5 keratin free condition.
3) Pre wash 1 dialysis unit per sample (Slide-A-Lyzer MINI Dialysis 3500

MWCO). Dialyze against 500mL of 50mM Ambic a few hours. Check no leak. Rinse 3 times with 50mM Ambic.
4) Wash carboxylase Ab and MAP2 Ab on Protein G beads from yesterday with 1x RIPA buffer first and wash with 50mM Ambic x 5 in keratin free condition.
5) Pre-heat PCR tubes with RapiGest solution (60*C, 2hrs) and rinse with 50mM Ambic x 5 keratin free condition.

Procedures:
1) Discard supernant from overnight NeutrAvidin pulldown, and Transfer beads to a low-protein binding tube.
2) Wash beads with 2% SDS x 2 (10 min each) at room temperature.
3) Wash beads with 1% TritonX100, 1% deoxycholate, 25 mM LiCl x2 (10 min each) at RT, Wash with 1 M NaCL x 2 (10 min each) at RT (500 uL each wash).
4) Wash with 50 mM Ambic x 5 (10 min each) @ RT (500 uL).
5) Rinse once with cold 0.1% RapiGest/2mM biotin/ 50 mM Ambic and transfer to PCR tubes prepared above.
6) Spin down in microcentrifuge, discard supernant, and add **40 uL** of 0.1% RapiGest/ 2mM biotin/50 mM Ambic.
7) Keep at 60*C for 2 hours.
8) Transfer to spin column (Pierce Spin Cups-Cellulose Acetate Filter) and spin down for 30 s at 3381xg in a low binding tube.
9) Transfer the flow-through to a low binding tube with 20 uL of HA beads and 6 uL of PC/MAP2 Ab conjugated beads.
10) Incubate overnight at 4*C (while shaking on nutator).

Day 3

Procedures:
1) Transfer sample to a spin column (Pierce Spin Cups-Cellulose Acetate Filter) with low binding tube and spin down for 30 sec at 3381xg.
2) Transfer the supernatant to a “Slide A lyzer MINI” and dialyze against 500 mL of 50mM Ambic twice (3 hours each).
3) Transfer to a low-protein binding tube.
4) Measure the protein concentration using BCA kit from Pierce.
5) Eluted protein is next lyophilized to dryness and resuspended in 20 uL of 1X SDS loading buffer supplemented with 10 mM dithiolthreitol.
6) All 20 µL is loaded onto an Invitrogen NuPAGE 4-12% SDS-PAGE gel and run for approximately 5 min to electrophorese all proteins into the gel matrix. The entire MW range was then excised in a single gel-band and subjected to standardized in-gel reduction, alkylation, and tryptic digestion.
7) Proceed to Mass Spectrometry.