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Brain Tissue Samples and Mitochondrial extracts Samples Western Blot

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We use this protocol and it's working

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Abstract

Brain Tissue Samples and Mitochondrial extracts Samples Western Blot used in the Mazmanian Lab, Caltech.

Striatal tissue samples protein extraction

1 Prepare 1x RIPA Lysis Buffer (Millipore, Cat #20-188) in PBS, adding one tablet of protease inhibitor (Sigma, Cat # 4693124001) per 50 mL. Add phosphatase inhibitor (Thermofisher, Cat #A32957) if testing for phosphorylated proteins.

2 Add 200uL of buffer to each sample

3 Homogenize tissue either using the bead beater (if doing 3 minutes, do 3 cycles of 1 minute beating, 1 min resting on ice because samples will get hot and degrade) or homogenizer or sonicator.

4 Extracts can be used immediately or stored in -80C freezer until needed.

Mitochondrial extracts protein extraction

5 After mitochondrial isolation, extracts are stored in -80C Freezer, which will extract mitochondrial proteins for further analysis.

Protein quantification

6 Prepare BSA (bovine serum albumin) standards following chart in Pierce BCA Protein Assay Kit (Thermofisher, Cat # 23209), diluting with PBS.

7 Follow Pierce BCA Protein Assay Kit for making other reagents – use instructions for plate/200uL . Samples can be diluted at 1:10 or 1:5 concentration in PBS before plating.

8 Add samples and reagents to plate and shake it for 30 seconds (

9 Place in incubator for 30 mins at 37C degrees.

10 Measure the absorbance at or near 562 nm on a plate reader

11 Normalize protein levels across samples for 5 ug- 40ug, using PBS. Need to keep in mind here that samples will be mixed with the same volume of 2 x Laemmli buffer, doubling the volume.

For isolated mitochondrial extract you will need more diluted samples (usually 5ug)

SDS-PAGE (gel):

12 Use Thermo Cycler Tubes to prepare samples.

13 Add same volume of 2 x Laemmli buffer (Biorad, Cat# 161-0737) to normalized protein extract.

14 Heat samples for 10 min at 98C using a Thermo cycler. If your protein is highly hydrofobic you may need to decrease heating temperature as well as the time (this is the case of some OXPHOS proteins). Check antibody datasheet.

15 While heating, set up gel 4-20%Tris gel (Invitrogen, Cat # XP04200BOX10). You may need different gels for different sized proteins.

16 Fill up inside of gel chamber about 2/3 of the way with 1x Tris-Glycine-SDS Running Buffer (TGS, Thermofisher, Cat#LC2675), outside chamber all the way up with TGS.

17 Load 5-7uL of protein ladder into first lane.

18 Carefully take out samples from incubator.

19 Carefully load samples into wells using proper technique, not puncturing gel or letting sample overflow. Use new pipette tip for each sample.

20 Connect lid, tightly place on top of blot box, and set gel to run for 1.5 hours at 140mV.

Protein Transfer (wet)

21 While gel is running, prepare for transfer.

22 Cut PVDF membrane (Millipore, Cat# IPVH00010) to approximate size of gel, making sure to never touch the membrane with hands or gloves. Only touch the blue liners and only use tweezers to pick up membrane.

23 Cut filter paper to approximate size of gel - need two per gel

24 Fill an empty pipette box with transfer buffer 1x Tris-Glycine buffer (transer buffer) and place 2 fat and 1 skinny sponges per gel plus your filter paper to soak. Make sure sponges are entirely soaked through with TG before doing transfer.

25 Fill an empty pipette box with about ¼ 100% methanol and place membrane in to activate for at least 10 minutes prior to transfer

26 Once gel is finished running, use metal spatula to crack open plastic around gel and trim gel to the size of the ladder

27 Make your blot "sandwich" in transfer box, using following items:
- Fat sponge
- Filter paper
- Gel
- Membrane
  - Be very careful not to touch membrane to the gel before it is properly aligned. Use tweezers and roller *only* to touch it and smooth out any air bubbles.
- Filter paper
- Fat sponge
- Skinny sponge

28 Insert transfer box into blot box and make sure it fits.

29 Fill outside chamber to the top with diH20 (just for insulation)

30 Fill outside chamber to the top with diH20 (just for insulation)

Protein detection

31 Take out membrane with tweezers, trim to ladder size if desired

32 Place in a box with blocking buffer (5% milk in TBS-20%Tween) to shake for 30min - 1 hr (or longer). Or 5% BSA in TBS-T or blocking buffer. We use TBS-T (Thermofisher, Cat #28358).

33 Add primary antibody 1:1000 ab:blocking buffer (or company recommended dilution for WB). Place in cold room shaking overnight.

34 Pipette off primary antibody.

35 Wash blot 3x for 5mins each, shaking, with TBS-T.

36 Add secondary antibody (IgG-HRP) 1:1000 in TBST-T and let shake at room temp for 1.5 hrs

37 Wash 3x for 5 mins each, shaking, with TBS-T

Imaging

38 Mix 1mL clarity reagent with 1mL hydrogen peroxide reagent from Clarity™ Western ECL Substrate (Biorad, Cat #1705060).

39 Turn on imager.

40 Place blot onto platform using tweezers, roll out any bubbles, and wipe excess reagent

41 Take images and save it.