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Western Blot V.1

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We use this protocol and it's working

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Abstract

TBD

Materials

Reagents:

- Tris (vendor, CAT#, URL)
- NaCl
- NP-40
- NaDeoxycholate
- SDS
- Protease Inhibitor cocktail tablet
- PhosSTOP tablet
- β-mercaptoethanol
- glycerol
- Bromophenol blue
- glycine
- MetOH
- Bovine Serum Albumin (BSA)
- chemiluminescent ECL Substrate Kit

Equipment:

- Tissue Tearor
- Spectrophotometer
- Electrophoresis machine

Preparing RIPA Buffer:

- 50 mM Tris pH8 0.606 g/100ml
- 150 mM NaCl 0.877 g/100ml
- 1 % NP-40
- 0.5 % NaDeoxycholate
- 0.1 % SDS

For each 10 mL add 1x Protease Inhibitor cocktail tablet and 1x PhosSTOP tablet.

Preparing Loading Buffer (6 X):

- 12 % SDS (1.2g / 10 ml)
- 30 % β-mercaptoethanol
- 60% glycerol
- 0.012 % Bromophenol blue
- 375 mM Tris pH 6.8

Preparing WB Running buffer (10X):

To make 1L:

- 144g glycine
- 30.3 g Tris base

- 10 g SDS
- MilliQ up to 1 L

Preparing TBS (10X):

To make 1L:

- 200 mL Tris 1 M pH 7.5
- 80g NaCl
- MilliQ up to 1L

To make TBST, add Triton at 1%.

Preparing Transfer buffer (10X):

To make 1L:

- 144g glycine
- 30.3 g Tris base

To dilute to 1X:

- 750 mL dH2O
- 150 mL MetOH
- 100 mL 10X Transfer buffer

Preparing Samples

1 Take samples out of the -80°C freezer and keep on dry ice until ready to digest.

2 On wet ice, add 200 µL RIPA Buffer (see **Materials**) to each unilateral striatum sample.

3 Mix thoroughly until sample completely blended with Tissue Tearor.

4 Centrifuge samples at 6g for 5 min at 4ºC.

5 Using the supernatant, dilute samples to about 1:10 to determine protein concentration.

Analysing Protein Concentration

6 **Prepare pre-diluted/standards:** 1.0, 0.8, 0.6, 0.4, 0.2 and 0.0 mg/mL of Bovine Serum Albumin (BSA)

7 **Prepare solution A:**
- 320 µL Copper (II) Sulfate Solution
- 16 mL Bicinchoninic Acid Solution

8 In a well plate, add 10 µL of pre-diluted samples/standards + 100 µL solution A.

Do this in triplicate.

9 Cover plates with film and incubate the samples for 30 min at 37ºC.

10 Measure the absorbance of each sample at 562 nm using a spectrophotometer and create a standard curve to determine concentrations.

Running Western Electrophoresis Gel

11 Make up 83 µL samples in RIPA Buffer at a concentration of 20 µg/10 µL and then add 17 µL of 6x Loading Buffer (see **Materials**).

12 Boil samples at this stage at 95 ºC for 5 min.

Note

Always check antibody data sheet before performing this step.

13 Prepare 1X Running Buffer (see **Materials**) and add the pre-cast gel cassettes (4 – 12%).

14 Load 5 µL of sample per well.

15 Load visual + developable ladder in first lane, and only developable ladder in last lane.

16 Run electrophoresis for 60 min @ 200V/100mA.

17 Transfer gel onto membrane and transfer using machine.

Running Antibodies

18 Wash membranes with TBST (see **Materials**) for approximately 1 min and repeat 4x.

19 Make up 50 mL TBST solution with 4% milk (dried powder).

20 Roll membranes into 50 mL conical with 3 mL of TBST w/ Milk and Primary Antibody.

The following primary antibody (working concentration 1:1000) was used in Brimblecombe, K. et al. (2023): anti-calb1 (Cell Signalling #13176).

Note

Primary Antibody Solutions can be re-used several times.

21 Incubate overnight at 4 ºC or ~2 hours at room temperature.

22 Wash membranes with TBST for approximately 1 min and repeat 4x.

23 Incubate membrane in TBST w/ Milk and Secondary Antibody HRP conjugated (1:3000) for 1 hour at room temperature.

Note

Secondary Antibody Solutions can be re-used several times.

24 Wash membranes with TBST for approximately 1 min and repeat 4x.

25 Develop membranes with the chemiluminescent ECL Substrate Kit (~ 1 mL of each component).

26 Visualize gel on a Gel Doc System.

27 Put membrane back into TBST w/ Milk and the 1º/2º β-actin antibody (already conjugated, 1:50000).

28 Repeat **steps 25 and 26**.