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Western blot in homogenised mouse brain samples V.2

In 1 collection

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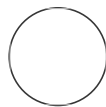
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ABSTRACT

This protocol is for western blot analysis of proteins in homogenised mouse brain samples. The sensitivity and selectivity of this assay is dependent on the efficacy of antibodies. When using novel antibodies ensure they have been validated for western blot, ideally using tissue from knock-out animals.

GUIDELINES

Due to the samples being homogenised, biological replicates should be from individual animals. Technical replicates run on repeated samples from the same animal can be illustrative of the reliability of each reading, however, they do not reflect biological variability.

MATERIALS

Reagents:

- Tris (vendor, CAT#, URL)
- NaCl
- NP-40
- NaDeoxycholate
- SDS
- Protease Inhibitor cocktail tablet
- PhosSTOP tablet
- β-mercaptoethanol
- glycerol

MANUSCRIPT CITATION:

Brimblecombe KR, Connor-Robson N, Bataille CJR, Roberts BM, Gracie C, O'Connor B, Te Water Naude R, Karthik G, Russell AJ, Wade-Martins R, Cragg SJ. Inhibition of striatal dopamine release by the L-type calcium channel inhibitor isradipine co-varies with risk factors for Parkinson's. Eur J Neurosci. 2023 Nov 8. doi: 10.1111/ejn.16180. Epub ahead of print. PMID: 37941514.

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We use this protocol and it's working

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92554

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- 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 MeOH
- 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 4°C.
- 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 μ L Copper (II) Sulfate Solution
 - 16 mL Bicinchoninic Acid Solution
- 8 In a well plate, add 10 μ L of pre-diluted samples/standards + 100 μ L solution A.
- Do this in triplicate.**
- 9 Cover plates with film and incubate the samples for 30 min at 37°C.
- 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 μ L samples in RIPA Buffer at a concentration of 20 μ g/10 μ L and then add 17 μ L of 6x Loading Buffer (see **Materials**).
- 12 Boil samples at this stage at 95 °C 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 μ L 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 °C 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 1°/2° β -actin antibody (already conjugated, 1:50000).
- 28 Repeat **steps 25 and 26**.