Western Blot

In 1 collection

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ABSTRACT

This protocol describes the solutions and steps for performing western blots.

PROTOCOL MATERIALS

SuperSignal™ West Pico PLUS Chemiluminescent Substrate Thermo Scientific Catalog #34577

Step 16

Pierce BCA Protein Assay Kit Thermo Fisher Scientific Catalog #23225

Step 5

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Preparation of lysate from tissues

25m

1 Dissect the tissue with clean tools, On ice preferably, and as quickly as possible to prevent degradation by proteases.

> **Note**
>
> *Striatum:*
> Using cold cutting solution, cut brain slices coronally with the vibratome in 240 µm steps until reaching the striatum, and then change the thickness to 500-600 µm to cover the whole striatum in one slice. Move the slice to a Petri dish with cutting solution On ice . Under the scope (keep the tray of the scope also On ice), use the scalpel and the forceps to dissect the striatum or its subregions.

2 Place the pieces of tissue microcentrifuge tubes and store samples at -80 °C for later use or keep on ice for immediate homogenization.

3 Add ice-cold lysis buffer rapidly to the tubes and homogenize with an electric homogenizer. Then denature the samples at 100 °C in a hot plate for 00:05:00 5m

> **Note**
>
> Volumes of lysis buffer must be determined in relation to the amount of tissue present. Optimal concentration of protein for loading gels is 1 – 5 mg/mL.

Note

Striatum:

- Lysis buffer: STE buffer: [M] 10 millimolar (mM) Tris-Cl pH 7.5, [M] 1 millimolar (mM) EDTA pH 8.0, [M] 1 % volume SDS

4 Centrifuge 12000 rpm, 4°C, 00:20:00 in a microcentrifuge. Remove the tubes from the centrifuge and place on ice, and use the cleared supernatant to determine protein concentration. 20m

Protein determination

5 Follow the manufacterer's instructions for protein determination (microplate assay).

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Electrophoresis (SDS-PAGE)

6 Gel preparation:

6.1

- Focusing gel (lower or bottom gel):

Prepare the focusing or resolving gel with the percentage or concentration required, depending on the size of your protein of interest. Gradient gels can also be used.

Protein size	Gel percentage
4 – 40 kDa	20%
12 – 45 kDa	15%
10 – 70 kDa	12.5%
15 – 100 kDa	10%
25 – 100 kDa	8%

	A	B	C	D	E
	Focusing gel	**7.5 %**	**10 %**	**12 %**	**15 %**
	Vf	10 mL	10 mL	10 mL	10 mL
	Acrylamide 30%	2.50 mL	3.33 mL	4.00 mL	5.00 mL
	1.5 M Tris pH=8.9	2.50 mL	2.50 mL	2.50 mL	2.50 mL
	10% SDS	100 uL	100 uL	100 uL	100 uL
	Water	4.79 mL	3.96 mL	3.29 mL	2.29 mL
	10% APS	100 uL	100 uL	100 uL	100 uL
	TEMED	10 uL	10 uL	10 uL	10 uL

6.2 ▪ Stacking gel (upper or top gel):

	A	B
	Stacking gel	
	Vf	5 mL
	Acrylamide 30%	670 µL
	0.5 M Tris pH=6.8	1.25 mL
	10% SDS	50 µL
	Water	2.99 mL
	10% APS	37.6 µL
	TEMED	7.5 µL

7 Loading and running:

7.1 Load equal and appropriate amounts of protein into the wells of the SDS-PAGE gel, along with molecular weight marker.

7.2 Run the gel for 1–2 h at 100 V (the time and voltage may require optimization, check the marker to obtain an optimal separation of the bands with the target molecular weight).

Transfer

8 Equilibrate gel and membrane in transfer buffer (membrane can be nitrocellulose or PVDF, previosuly activated with methanol for 1 min and rinsed in transfer buffer) .

9 Assemble gel and membrane sandwich: place gel and membrane between buffer-soaked filter papers and sponges. Gently remove air bubbles.

10 Perform transfer: place transfer assemble in transfer cell and fill with buffer. The time and voltage of transfer require some optimization.

Immunodetection

2h 10m

11 Block the membrane for 01:00:00 at Room temperature or overnight at 4 °C 1h using blocking buffer ([M] 5 % volume milk in TBS-T 0.1% Tween).

12 Incubate the membrane with appropriate dilutions of primary antibody in blocking buffer at 4 °C overnight.

13 Wash the membrane in three washes of TBS-T, 00:05:00 each. 5m

14 Incubate the membrane with the recommended dilution of horseradish peroxidase HRP-conjugated secondary antibody in blocking buffer 01:00:00 Room temperature 1h

15 Wash the membrane in three washes of TBS-T, 00:05:00 each. 5m

16 For signal development, follow the kit manufacturer's recommendations:

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17 Acquire image using darkroom development techniques for chemiluminescence.

18 If stripping and reprobe is needed:

18.1 Incubate membrane with stripping buffer up to 00:10:00 10m

18.2 Wash the membrane in three washes of TBS-T, 00:05:00 each. 5m

18.3 Repeat steps from 11 to 17 with different antibodies or dilutions.

Analysis

19 Perform densitometry analysis of the images in Fiji (ImageJ).

Buffers

20 **10X STE buffer**

- 100 mM Tris-Cl: pH=7.5
- 10 mM EDTA pH=8.0
- 10% SDS.

10X TBS (Tris-buffered saline)

- 200 mM Tris.
- 1500 mM NaCl.
- pH=7.6 with 12 N HCl.

1X TBS

- 20 mM Tris.
- 150 mM NaCl.
- pH=7.6 with 12 N HCl.

TBS-T (Tris-buffered saline, 0.1% Tween-20)

- 20 mM Tris.
- 150 mM NaCl.
- 0.1% Tween-20.
- pH=7.6 with 12 N HCl.