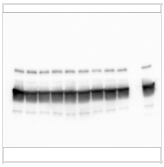

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Western for Brain Punches

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1 Works for me This protocol is published without a DOI.

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ABSTRACT

This protocol outlines the step-by-step procedure of a western blot; for isolating and probing for proteins from a rat brain punch.

PROTOCOL CITATION

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Western Blot

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GUIDELINES

- Tissue samples need to be kept on ice ro prevent degradation by proteases.
- Lysis buffer (with inhibitors) should be ice-cold prior to homogenization.
- Extra caution should be taken to prevent introduction of air bubbles anywhere between the filter paper, gel or membrane.
- If using PVDF membrane: prewet membranes in methanol.
- Use BSA instead of milk, if using antibodies that target phosphoproteins.

MATERIALS TEXT

MATERIALS

SDS **BIO-RAD Catalog #161-0302**

Blotting Grade Blocker **BIO-RAD Catalog #170-6404** Step 28

PBS **Contributed by users**

Glycine **Sigma Catalog #50046**

Triton X-100 **Fisher Scientific Catalog #85112**

Methanol (MeOH) **Fisher Scientific Catalog #10499560**

Sodium deoxycholate (SDC) **Sigma Aldrich Catalog #30970**

BSA **Sigma Aldrich Catalog ##A8806**

Tween-20 **Sigma Aldrich Catalog #P9416**

Sodium chloride **Sigma Aldrich Catalog #S3014**

Novex™ Tris-Glycine Transfer Buffer (25X) **Thermo Fisher Catalog #LC3675**

Sodium Azide **Sigma Aldrich Catalog #S2002-100G**

Tris base **Sigma Aldrich Catalog #T1503**

Recipes

RIPA buffer (radioimmunoprecipitation assay buffer)

- 150 mM sodium chloride
- 1.0% NP-40 or Triton X-100
- 0.5% sodium deoxycholate*
- 0.1% SDS (sodium dodecyl sulfate)
- 50 mM Tris, pH 8.0

25X Novex Tris-Glycine Transfer Buffer: (Novex apparatus only)

- Dissolve the following in ~400mL ddH2O
- Tris Base 18.2g (12mM final concentration)
- Glycine 90.0g (96mM final concentration)
- Adjust Volume to 500mL with ddH20
- The pH of 1X buffer is 8.3. Do not adjust with acid or base.

1X Novex Tris-Glycine Transfer Buffer: (Novex apparatus only)

- 40mL 25X Novex Tris-Glycine Transfer Buffer
- 200mL MeOH (final 20% v/v)
- ddH20 to 1L
- *Must stay cold, store in cold room*

PBST: (0.05% Tween-20 in PBS)

- 1L:100mL PBS
- 900 mL ddH20
- 0.5mL Tween-20
- *Anything with alkaline phosphatase, must use TBST instead

Blocking Solution: (5% (w/v) milk in PBST)

- *Usually need 10mLfor block, 10mL for secondary, PER BLOT)
- 2g milk powder
- 40mL PBST
- *Stores for up to a week at 4°C
- *Anything with Biotin, cannot use milk blocking

BSA Solution: (3% (w/v) Bovine Serum Albumin in PBST)

- *Usually need ~5-10mL for wash, 10mL for antibody unless already made, PER BLOT)
- 0.6g BSA
- 20mL PBST

Primary Antibody Solution

- 3% BSA

- 0.05% Na-Azide
- *Can be reused many times, keep at 4°C

SAFETY WARNINGS

Basic lab safety required

ABSTRACT

This protocol outlines the step-by-step procedure of a western blot; for isolating and probing for proteins from a rat brain punch.

BEFORE STARTING

- Cool down centrifuge
- Make sure transfer buffer is made and in the fridge
- Make sure miliQ water is in the fridge

Cell Lysis & Protein Extraction

1 Make lysis solution
Add RIPA an 100X HALT (for end solution of 1X HALT)

Halt™ Protease and Phosphatase Inhibitor Cocktail (100X) **Thermo Fisher Catalog #78440**

2 Add **20 µl** RIPA/HALT solution to brain punch.

3

Homogenize with micropipette (by pipetting up & down and avoiding bubbles).

4

Spin the samples in cold centrifuge at **6000 rpm, 4°C, 00:10:00**

Eppendorf 5415R Refrigerated Centrifuge
Refrigerated Centrifuge

Eppendorf **EP-5415R**

Sample Preperation

5

Make a sample buffer

45 µl 4X Laemmli Sample Buffer + **5 µl** 2-Mercaptoethanol.

4x Laemmli Sample Buffer **BioRad Sciences Catalog #1610747**

2-Mercaptoethanol **BioRad Sciences Catalog #1610710**

6 After centrifugeing sample from Part 1, put them **On ice**

7

Take 200 ul strip tubes and label. Add **5 µl** of the sample buffer and **15 µl** of the sample in each tube.

7.1 Repalce strip tube lid.

8

Incubate these samples in the PCR machine for **00:05:00** at **95 °C**

C1000 Touch Thermal Cycler
PCR Thermal Cycler
BioRad Sciences **C1000**

Running Gel

9 Take a BioRad gel (with 12 wells if you have 10 smapels) from the fridge. Pull gently to remove the green tape from the bottom of the cassette, and remove the green comb by pulling upward in one smooth motion.

Mini-PROTEAN Precast Gels **BIO-RAD**

10 Insert the gel in the BioRad electrophoresis chamber (with the long side of the gel facing you) and insert the wall on the other side (with the solid side towards the wire).

Mini-PROTEAN Tetra Vertical Electrophoresis Cell
lectrophoresis Chambers

BIO-RAD 1658005

11 Pour the running buffer in the middle and let it overflow until the solution reaches the black line at the bottom.
10x Tris/Glycine/SDS **Bio-rad Laboratories Catalog #1610732**

12 Clean wells with a 10 ml syringe and 17 G needle. Denser fluid may be at bottom of wells and needs to be flushed out.

13 Take samples out of PCR machine.

14 Using gel loading tips, add **10 µl** ladder to first and last well.

Precision Plus Protein™ Dual Color Standards **BIO-RAD Catalog #1610374**

15 Add **15 µl** each sample to each well to bottom of wells.

16 Replace top to electrophoresis chamber (*red on left with gel closer to you*).

17 Run for **00:30:00** at 200 V.

PowerPac Basic Power Supply
Power Supply

Bio-Rad Scientific 1645050

Transfer

18 Be sure that transfer buffer and milliQ water for transferring is cold 4 °C .

19 Rinse transfer pads with deionized water.

20 Place transfer pads and filter paper into transfer container and soak in transfer buffer on shaker.

21 Open the gel cassette using tool

21.1 Cut off one corner of the gel with a clean sharp razor blade or scalpel to allow you to orientate the gel.

21.2 Cut off top wells, bottom, and sides to remove gel excess.

22 ⚠

Assemble blot module.

XCell SureLock™ Mini-Cell and XCell II™ Blot Module
Blot Module
ThermoScientific EI0002

22.1 On anode (+) core place 2 transfer pads, roll the air out with falcon tube and add more transfer buffer on top.

22.2 Add 1 filter paper, roll out air, and more transfer buffer on top

22.3 Add 1 membrane

22.4 Cut off one corner of the gel mark first well location

22.5 Layer gel on top, preventing air from being trapped under

22.6 Add 1 filter paper on top of gel, 3-4 transfer pads, roll out air after each one, and add more transfer buffer on top.

22.7 Place the ???????cathode (-) core on top of the pads.
- The gel/membrane sandwich should be held securely between the two halves of the blot module ensuring complete contact of all components.

23 Hold the blot module together firmly and slide it into the guide rails on the lower buffer chamber.
Note: blot module fits into the unit in only one way.

24 Add transfer buffer inside blot module and milliQ water in surrounding area.

25 ⚠

Run transfer for **01:30:00** min at 24 V.

Do not let sit for an extended period of time after transfer has been completed

26 During transfer, make 3% BSA solution.

27 Upon completion of transfer, remove gel (throw away) and put membrane right-side up in a container (e.g., empty micropipette tip container).

28 Add blot blocker (blocking milk) and leave membrane on shaker for a minimum of **01:00:00**

Blotting Grade Blocker **BIO-RAD Catalog #170-6404**

Primary Antibody

29 Drain blot blocker from membrane and give a quick rinse with PBST.

30 Wash membrane in small amount of BSA solution.

31 Cut top and bottom off membrane around the area of interest, as well as in between the protein of interest and the housekeeping reference protein.

32 Add each piece of membrane to separate Petri dish.

33

Pipette 1° antibody into each Petri dish. Incubate **Overnight** at **4 °C** .

Secondary Antibody

34

Pipette out 1° antibody from Petri dishes.
(be sure to add the antibodies to their respective tubes for future use)

35 Wash in PBST once quickly by hand to remove excess 1° antibody.

36 Wash in generous amount of PBST for 5 min 3 times on shaker (*some antibodies may need more washing*).
Wash 1: **00:05:00**
Wash 2: **00:05:00**
Wash 3: **00:05:00**

37 Dilute 2° antibodies with horseradish peroxidase to 1:10,000 (1 ul antibody in 10 ml milk) in falcon tubes or as directed in milk blocking solution.

38 Lightly dry membrane strips and place on parafilm (away from workbench edge).

39 Pipette diluted 2° antibody to membrane strips.

40 Incubate for **00:45:00** at **Room temperature** on a stationary surface.

41 Wash in PBST once, quickly by hand.

42 Wash in PBST for 5 min 4 times on shaker.
Wash 1: **00:05:00**
Wash 2: **00:05:00**

Wash 3: **00:05:00**

Wash 4: **00:05:00**

Imaging

43 Transport blot in Petri dish with PBST for imaging.

44

Open “Image Lab 5.2.1" and set up imaging parameters.
1. Select *Chemi* under Application
2. Press *Signal Accumulation Setup.*
3. Set last image to 900 sec and 15 sec between each image (60 images total).

45 Drain PBST from Petri dishes.

46 Add oxidizing reagent and equal amount of enhancing reagent to cover membrane.

Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate **Contributed by users Catalog #NEL105001EA**

47 Manually shake for **00:02:00** to ensure the whole blot is covered.

48 After a light dry, put membrane strips into a single piece of binder sheet protector plastic.

49 Lay inside imager and adjust position using camera to center.

50 Run program until an good signal occurs for each blot.

51 Save the photo just prior to oversaturation for each blot.

52 Turn off imager and computer.

Stripping & Reprobing

53 Drain PBST from Petri dishe.

54 Wash membrane in 1X TBS once, quickly by hand.

55 Wash membrane in 1X TBS for 5 min 3 times on shaker.
Wash 1: **00:05:00**
Wash 2: **00:05:00**
Wash 3: **00:05:00**

56 Drain TBS and remove excess liquid from Petri dish with Kimwipe

57 Immerse blot in Restore Western Blot Stripping Buffer and incubate on shaker for **00:10:00** .

Restore™ Western Blot Stripping Buffer **Thermo Fisher Catalog #21059**

58 Drain Restore Western Blot Stripping Buffer from Petri dishes.

59 Wash membrane in 1X TBS for 5 min 5 times on shaker.
Wash 1: **00:05:00**
Wash 2: **00:05:00**
Wash 3: **00:05:00**
Wash 4: **00:05:00**
Wash 5: **00:05:00**

60 Add blot blocker (blocking milk) and leave membrane on shaker for a minimum of **01:00:00**

61 Continue with protocol for primary and secondary antibodies for second protein of choice
Follow protocol through imaging