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Western Blot

In 1 collection

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We use this protocol and it's working

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Abstract

This protocol describes the standard western blot (immunoblot) technique for detecting specific proteins in a complex sample. The procedure involves separating proteins by molecular weight using SDS-PAGE, transferring them to a membrane, and probing with target-specific antibodies to achieve selective detection. The method provides a reliable approach for protein analysis, including quantification of expression levels and verification of molecular weight.

Guidelines

DTT recipe: 0.25 M Tris-HCl pH 6.8, 200mM DTT, 8% SDS, 30% glycerol, 2mg bromophenol blue

Materials

- BCA protein assay and standards ready concentrations referenced: 2000, 1500, 750, 500, 250, 125 and 0 µg/µL
- PBS
- Kit buffers (prepare mixed at 1:50 ratio; add 100 µL to samples)
- Dryboil plate (for boiling samples)
- Heating block or dry plate capable of 95°C
- 15% acrylamide SDS-PAGE gels (cassettes)
- Running buffer
- Transfer buffer
- Nitrocellulose membrane
- Transfer sandwich materials: 2 sponges, 1 paper filter (per side), gel, nitrocellulose membrane
- Tweezers (for transfers)
- Containers for transfer at 100 V (power supply)
- PBS for rinsing and fixation
- 0.4% PFA in PBS (for fixation)
- TBST (Tris-buffered saline with Tween)
- 5% milk (blocking solution)
- Primary antibodies: BD anti-asyn (Cat# 610787; mouse) at 1:1000; GAPDH (Cat# 14C10; CS rabbit) at 1:10,000
- Secondary antibodies: anti-mouse HRP and anti-rabbit HRP, each used at 1:2000 in 5% milk
- ECL solution (prepare 1:1 mixture; ~2 mL per blot)
- Chemidoc (or equivalent imaging system)
- Kimwipes or absorbent paper
- Sheet protector (to place membranes on before applying ECL)

Safety warnings

- *Listen out for popping of caps* when boiling samples and do a quick spindown afterwards.
- *Do not ever touch filters directly, use tweezers for transfers.*
- *Always check to make sure the buffer is bubbling/current is flowing* during electrophoresis and transfer.
- *Roll out any bubbles* when assembling the transfer sandwich; ensure no bubbles remain between gel and membrane.
- Note about antibody reuse: *Can save Ab! Store in a conical tube in the freezer. Can be used up to 3–4 times before Ab starts to wear off.*
- During last wash before secondary incubation: prepare secondary antibody and let it mix on shaker until ready.

Before start

- Ensure ready-to-use BCA standard concentrations are prepared: 2000, 1500, 750, 500, 250, 125 and 0 µg/µL. add 5 µL of standards (no PBS) in triplicate in set wells. Dilute samples at 1:5 (4 µL PBS : 1 µL sample) in triplicate when needed, then add 100 µL of BCA kit buffers mixed at 1:50 ratio.
- For sample prep: target 20 µg protein in 10 µL sample;
- Prepare gels, running buffer, transfer buffer, nitrocellulose membranes, and transfer sandwich components in advance.
- Prepare blocking solution (5% milk) and TBST; pre-chill primary antibody mix if incubating overnight at 4°C.

Day 1

1 Prep samples for BCA assay: *add in 5 µL of standards, no PBS. Ready-to-use concentrations should already be made up (2000, 1500, 750, 500, 250, 125 and 0 µg/µL). dil*ute samples at 1:5 (so 4 µL PBS : 1 µL sample), then add 100 µL of the kit buffers mixed at a 1:50 ratio.

2 make samples: 20 µg protein in 10 µL volume with 1XDTT for denaturing.

Day 2

3 Boil samples at 95°C for 10 minutes in a dryboil plate *listen out for popping of caps, do a quick spindown afterwards*.

4 Load samples (15% acrylamide gels) and run gel in running buffer *put gels in cassettes facing inwards. Make note of which samples align with which well in the gels*

5 Run at 80 V until samples leave the stacking layer, then run at 100 V until samples are at the bottom of the gel. *Always check to make sure the buffer is bubbling/current is flowing*.

6 Transfer samples in transfer buffer onto a nitrocellulose membrane at 100 V for 1 h.

6.1 Assemble transfer sandwich in the following order (from bottom to top):
Black side of comb on bottom
2 sponges
1 paper filter
Gel
Nitrocellulose membrane
1 paper filter
2 sponges

7 Roll out any bubbles! *do not ever touch filter directly, use tweezers for transfers*.

8 When transfer is done, isolate membrane.

9 Rinse blots in PBS, fix in 0.4% PFA for 30 minutes, then wash with TBST (3 quick rinses).

10 Block membranes in 5% milk for 60 minutes at room temperature on a shaker.

11 Incubate membranes in primary antibody overnight at 4°C in 5% milk.
can save Ab! Store in a conical tube in the freezer. Can be used up to 3-4 times before Ab starts to wear off.

7.3.25

12 Wash blot in TBST 3 × 5 minutes. *during last wash, make 2º Ab and let mix on shaker until ready.*

13 Incubate in secondary antibody for 1 hour at room temperature with shaking.

14 Wash blots 3 × 5 minutes in TBST.

15 Prepare ECL solution (1:1 mixture, ~2 mL per blot) and image on the Chemidoc.

16 *Place membranes on a sheet protector and apply ECL solution ALL over the blot.*
Incubate for 2 min in ECL and make sure there are no bubbles.
Make a pile of Kimwipes and lay them on top of blots to absorb ECL before taking pictures on the Chemidoc.