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Western Blot

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ABSTRACT

Western blot is a technique used to separate proteins by size followed by detection using antibodies specific to the protein of interest. This protocol describes the basic steps for lysing cells, determining total protein concentration in the lysate, running a precast SDS-PAGE gel, and immunoblotting.

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GUIDELINES

General Considerations

Acrylamide percentage varies in SDS-PAGE gels. Use lower percentage acrylamide when immunoblotting high molecular weight proteins and higher percentage acrylamide when immunoblotting low molecular weight proteins.

This protocol uses a dry transfer device but can be adapted for wet and semi-dry transfer approaches.

Workflow Timeline

Day 1: Prepare lysates, run SDS-PAGE, transfer, block, incubate with primary antibody
Day 2: Incubate with secondary antibody

Tips and Troubleshooting:

The optimal lysis buffer will vary depending on the sample type and cellular location of the protein of interest. You may need to try a variety of lysis buffers to find the best for your target.

The optimal concentration and blocking buffer will vary between antibodies. Review the instructions before starting your experiment and consider titrating your antibody to determine the optimal dose.

To ensure that your antibody is both functioning as expected and is specific, include a positive control sample that you know expresses the protein, such as cells transfected with the protein of interest, and a negative control sample that does not express the protein of interest.

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MATERIALS TEXT

Equipment:

- Microcentrifuge
- 0.5–10 µL single channel pipette
- 2–20 µL single channel pipette
- 20–200 µL single channel pipette
- 200–1000 µL single channel pipette
- Pipette controller
- Pipette tips and pipettes
- Spectrophotometer
- Heat block
- Mini gel tank chamber
- Power supply
- iBlot 2 Gel Transfer Device
- Roller
- Spatula
- Platform shaker
- Cold room
- Gel imager
- -80 °C freezer

Reagents and Consumables:

- 1X PBS
- Lysis buffer e.g., RIPA lysis buffer
- Microcentrifuge tubes
- BCA Assay, Thermo Fisher 23227
- β-mercaptoethanol
- 4X protein loading buffer
- Precast SDS-PAGE gel
- SDS-PAGE running buffer
- Prestained protein ladder
- Ethanol
- iBlot 2 PVDF Mini Stack, Thermo Fisher IB24002
- 20X TBS
- Tween-20
- Nonfat milk powder
- 96-well microtiter plate
- Chemiluminescence substrate
- Plastic wrap
- Primary antibody
- Secondary antibody
- Deionized water

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BEFORE STARTING

Refer to the manufacturer's instructions for additional information specific to your antibody, such as ideal blocking buffer and optimal antibody concentrations. Consider titrating your antibody to determine the optimal dose.

Secondary antibodies must match the host species of the primary antibody. For example, use an anti-mouse secondary antibody for primary antibodies raised in a mouse.

Lyse cells

5m

1 Centrifuge 0 µL for 00:05:00 at 100 x g.

2 Gently remove supernatant.

3 Resuspend cell pellet in 1 mL of 1X PBS and transfer to a microcentrifuge tube.

5m

4 Centrifuge for 00:05:00 at 100 x g.

5 Carefully remove supernatant.

6 Resuspend cell pellet in appropriate volume of cold lysis buffer.

Note

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The volume of lysis buffer will vary depending on the size of your cell pellet but will generally be between 250–1000 µL.

Note

The ideal lysis buffer will vary depending on the cellular location of the protein of interest. RIPA buffer is suitable for most proteins but more stringent buffers and a sonication step may be required for hard to extract proteins such as those in the nucleus.

7 Incubate on ice for 00:30:00. 30m

8 Centrifuge lysate for 00:15:00 at 14.000 x g at 4 °C. 15m

9 Transfer supernatant to a clean microcentrifuge tube.

10 Use the lysate immediately or store at -80 °C until ready to use.

Determine the total protein concentration and prepare the lysate for

11 Determine the protein concentration using a Pierce BCA Assay Kit or other preferred method for protein determination.

11.1 Prepare a 50:1 Reagent A to Reagent B dilution of the BCA assay.

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11.2 Prepare serial dilutions of the BSA standard that range from 0–2000 µg/mL.

11.3 In duplicate, dilute 10 µL of standard, blank, and lysate samples into 200 µL of BCA reagent in a 96-well microtiter plate.

11.4 Incubate for 00:30:00 at 37 °C. 30m

11.5 Determine the absorbance at 590 nm.

11.6 Calculate the average absorbance of the duplicate samples on the plate.

11.7 Subtract the average absorbance of the blank from all of the samples.

11.8 Plot a standard curve of BSA standard concentration versus absorbance.

11.9 Extrapolate the total protein concentration of the sample from the standard curve.

12 Determine the volume of sample required to load an equivalent amount of total protein for each sample.

Note

The ideal total protein loaded will vary between samples and target proteins but is typically between 10–50 µg. If the protein is in low abundance in the sample you will need to load a greater amount of total protein.

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13 Prepare sample for loading as follows:

13.1 Add 10% v/v β-mercaptoethanol to the 4X protein loading buffer.

13.2 Dilute 4X protein loading buffer in the sample to 1X.

14 Boil the samples for 00:10:00 at 100 °C. 10m

SDS-PAGE

15 Prepare the precast gel as follows:

15.1 Remove the gel from the plastic packaging.

15.2 Remove tape and plastic comb.

15.3 Rinse the wells with deionized water 3x.

15.4 Shake the gel gently between washes to remove residual water.

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15.5 Load the gel into the chamber of the SDS-PAGE gel tank.

> Note
>
> Different SDS-PAGE gel tanks will have different gel orientations. Refer to the instructions for your specific tank.

15.6 Close the clamp.

16 Prepare 1X running buffer as follows:

16.1 Dilute 25 mL of 20X running buffer to 500 mL with deionized water. Mix well.

17 Fill the chamber of the SDS-PAGE gel tank with the 1X running buffer.

18 Load the samples into the gel.

19 Load 5–10 µL of the prestained protein ladder.

20 Place the lid on the tank and plug the electrode cords into the power supply.

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21 Run the gel at **100 V** for 10–15 min or until the samples have moved out of the wells and into the gel.

22 Increase the voltage to **150 V** and continue running the gel until you have obtained the desired separation.

23 Gently open the gel with the spatula.

Dry transfer

15m

24 Soak the gel for 00:15:00 in 20% ethanol in deionized water.

24.1 To prepare 20% ethanol, dilute 2 mL of ethanol into 8 mL of deionized water and mix well.

25 Assemble the transfer sandwich as follows:

25.1 Unseal the iBlot 2 PVDF Mini transfer stack.

25.2 Set the Top Stack to one side and discard the white separator.

25.3 Keep the Bottom Stack in the plastic tray.

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25.4 Place the Bottom Stack on the blotting surface.

25.5 Align the electrical contacts on the blotting surface of the iBlot 2 Gel Transfer Device.

25.6 Wet the pre-run gel in deionized water and place it on the transfer membrane of the bottom stack.

25.7 Soak a piece of iBlot Filter Paper in deionized water.

25.8 Place the pre-soaked iBlot Filter Paper on the gel and remove the air bubbles with the roller.

25.9 Place the Top Stack over the pre-soaked filter paper.

25.10 Remove air bubbles using the roller.

25.11 Place the Absorbent Pad on top of the Top Stack such that the electrical contacts are aligned with the corresponding electrical contacts on the blotting surface of the iBlot 2 Gel Transfer Device.

25.12 Close the lid of the device.

25.13 Select the desired method and make sure the parameters are correct.

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25.14 Transfer for 5–6 min for proteins <30 kDa.

25.15 Transfer for 8–10 min for proteins >150 kDa.

25.16 Select Start Run.

26 When the run is complete, select Done.

Blocking

27 Prepare 1X TBST as follows:

27.1 25 mL of 20X TBS

27.2 2.5 mL of Tween-20

27.3 472.5 mL of deionized water

27.4 Mix well

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28 Prepare blocking buffer as follows:

28.1 Dilute 5% w/v non-fat milk into 1X TBST. (Example: 5 g of non-fat milk into 100 mL of 1X TBST.)

> **Note**
>
> The ideal blocking buffer will vary between antibodies. Refer to the manufacturer's information before running your experiment.

29 Remove the membrane from the transfer device and place it in a container with 1X TBST, protein side up.

30 Block the membrane in blocking buffer for 01:00:00 at Room temperature on a shaking platform. 1h

31 Wash the membrane 3x for 00:05:00 in 1X TBST at Room temperature on a shaking platform. 5m

Antibody incubation 1h 15m

32 Dilute the primary antibody to the desired concentration in blocking buffer.

> **Note**
>
> The optimal concentration will vary between antibodies but is typically between 1–10 µg/mL.

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33 Incubate the membrane overnight in primary antibody at 4 °C on a rocking platform.

> Note
>
> Primary antibody incubation can be reduced to 02:00:00 at Room temperature but may result in more non-specific binding.

34 Wash the membrane 3x for 00:05:00 in 1X TBST at Room temperature on a shaking platform. 5m

35 Dilute the horseradish peroxidase-conjugated secondary antibody to the desired concentration in blocking buffer.

> Note
>
> The optimal concentration will vary between antibodies but is typically between 1–10 µg/mL.

36 Incubate the membrane with secondary antibody for 01:00:00 at Room temperature on a shaking platform. 1h

37 Wash the membrane 3x for 00:05:00 in 1X TBST at Room temperature on a shaking platform. 5m

38 Prepare the chemiluminescence substrate by mixing 1:1 reagent A to reagent B.

39 Gently incubate the membrane in the chemiluminescence reagent for 00:05:00 at Room temperature. 5m

Cover the membrane with clear plastic and use a gel imager with chemiluminescence detection or a dark room

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40 to detect the bands.

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