General procedure for fluorescent western blotting

Materials

- Nitrocellulose or PVDF transfer membrane (e.g., Thermo Scientific™ membranes, Cat. No. 88018 or 22860, or equivalent)
- Transfer buffer (e.g., Invitrogen™ NuPAGE™ Transfer Buffer, Cat. No. NP0006; Invitrogen™ Novex™ Tris-Glycine Transfer Buffer, Cat. No. LC3675)
- Wash buffer, Tris-buffered or phosphate-buffered saline with 0.05% Tween™ 20 surfactant (e.g., Thermo Scientific™ Pierce™ 20X TBS Tween™ 20 Buffer, Cat. No. 28360; Thermo Scientific™ Pierce™ 20X PBS Tween™ 20 Buffer, Cat. No. 28352)
- Filtered blocking buffer (e.g., Thermo Scientific™ Blocker™ FL Fluorescent Blocking Buffer, Cat. No. 37565)
- Incubation trays and containers
- Primary antibodies (e.g., Invitrogen™ western blot–validated* primary antibodies)
- Secondary antibodies (e.g., Invitrogen™ fluorescently labeled highly cross-adsorbed secondary antibodies)

Protocol

1. Prepare transfer buffer for wet or semi-dry transfer based on gel chemistry.
2. Prepare transfer membrane. For dry transfer, follow manufacturer's instructions for preparation of membrane.
   - PVDF: pre-wet in methanol or ethanol (100%) for 30 seconds, briefly rinse in deionized water and equilibrate in transfer buffer for 5 minutes.
   - Nitrocellulose: equilibrate directly in transfer buffer for 5 minutes.
3. Follow manufacturer's instructions for wet, semi-dry, or dry transfer.

Suggested transfer devices

- **For wet transfer: Invitrogen™ Mini Blot Module**
- **For semi-dry transfer: Invitrogen™ Power Blotter System**
- **For dry transfer: Invitrogen™ iBlot™ 2 Gel Transfer Device**

4. After protein transfer, wash the membrane in deionized water 4 times for 5 minutes each with agitation to remove all transfer buffer.
5. Incubate the membrane with a sufficient volume of blocking buffer for 30–60 minutes at room temperature with agitation.

Tips:

Do not add detergent to blocking buffer, as this may increase background fluorescence.

For typical incubation trays, use at least 15 mL for mini blots and 30 mL for midi blots to fully cover the membrane. Avoid low volumes, as differences in agitation and coverage can produce high or uneven background.

6. Dilute the primary antibody per supplier recommendations in the blocking buffer.

7. Incubate the membrane protein-side up in the primary antibody solution with agitation, for 1 hour at room temperature or overnight at 2–8°C. Ensure the volume of the antibody solution is enough to fully cover the membrane.

8. Wash the membrane 3 times with agitation for 10 minutes each in wash buffer. (If using a fluorescently conjugated primary antibody, proceed to step 11.)

9. Prepare dilutions of the conjugated secondary antibody ranging from 0.4 to 0.1 µg/mL in an appropriate volume of wash buffer, or alternatively in blocking buffer. From a 2 mg/mL antibody stock, dilute 1:5,000 to 1:20,000:

    - 1:5,000: 3 µL of secondary antibody in 15 mL wash buffer
    - 1:10,000: 1.5 µL of secondary antibody in 15 mL wash buffer
    - 1:20,000: 0.75 µL of secondary antibody in 15 mL wash buffer

10. Incubate the membrane protein-side up in the secondary antibody solution for 1 hour with agitation at room temperature. Ensure the volume of the antibody solution is enough to fully cover the membrane. Protect the membrane from bright light to prevent photobleaching of the fluorescent dyes.

11. Wash the membrane 6 times with agitation for 5 minutes each in wash buffer to remove any unbound secondary antibodies. It is crucial to thoroughly wash the membrane at this step. Protect the membrane from bright light to prevent photobleaching of the fluorescent dyes.

Tips:

The final wash time may be reduced by filling and decanting the tray with distilled water 4 times, then moving forward with three 5-minute washes in wash buffer.

12. Blots can be imaged immediately while still wet, or alternatively may be dried prior to imaging. To prevent contamination, place the blot in a sheet protector or on a clean surface prior to imaging.

Tips:

To dry the membrane, place it between two sheets of western blot filter paper to protect it from light exposure while drying. Drying the membrane allows for extended storage of the blot and can reduce exposure times. Store blots in the dark to prevent photobleaching.

*The use or any variation of the word "validation" refers only to research use antibodies that were subject to functional testing to confirm that the antibody can be used with the research techniques indicated. It does not ensure that the product(s) was validated for clinical or diagnostic uses.

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