General procedure for chemiluminescent western blotting

Materials

- Nitrocellulose or PVDF transfer membrane (e.g., Thermo Scientific™ membranes, Cat. No. 88018 or 88518, 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)
- Blocking buffer (e.g., Thermo Scientific™ Pierce™ Clear Milk Blocking Buffer, Cat. No. 37587)
- Incubation trays and containers
- Primary antibodies (e.g., Invitrogen™ western blot–validated* primary antibodies)
- Secondary antibodies (e.g., Invitrogen™ western blot–validated* HRP antibodies)
- Chemiluminescent HRP substrate (e.g., Thermo Scientific™ SuperSignal™ West Pico PLUS substrate, Cat. No. 34580; SuperSignal™ West Atto Ultimate Sensitivity Substrate, Cat. No. A38555)

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.

6. Dilute the primary antibody per supplier recommendations (Table 1) in the blocking buffer.

Table 1. Recommended primary and secondary antibody dilutions to use with Thermo Scientific™ chemiluminescent substrates.

	Recommended primary antibody dilution	Recommended secondary antibody dilution
Pierce ECL	1:1,000 (0.2–10 µg/mL)	1:1,000–1:15,000 (0.07–1.0 µg/mL)
SuperSignal West Pico PLUS	1:1,000 (0.2–1.0 µg/mL)	1:20,000–1:100,000 (10–50 ng/mL)
SuperSignal West Dura	1:5,000 (0.02–1.0 µg/mL)	1:50,000–1:250,000 (4–20 ng/mL)
SuperSignal West Femto	1:5,000 (0.01–0.2 µg/mL)	1:100,000–1:500,000 (2–10 ng/mL)
SuperSignal West Atto	1:5,000 (0.2–1.0 µg/mL)	1:100,000–1:250,000 (4–10 ng/mL)

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.

9. Dilute the conjugated secondary antibody in an appropriate volume of wash buffer or alternatively in blocking buffer (Table 1).

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.

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.

12. Prepare the working solution of chemiluminescent substrate based upon the manufacturer's instructions. Suggested volume is ~8–10 mL for mini blots and 15 mL for midi blots (0.1 mL working solution per cm^2 of membrane).

13. Incubate the blot with the working solution for 1 minute when using standard ECL substrates or 5 minutes when using high-performance substrates, such as Thermo Scientific™ SuperSignal™ substrates.

14. Remove the blot from the working solution and drain excess reagent.

15. Place the blot in clear plastic wrap or a sheet protector and remove bubbles by rolling with a blot roller or a pipette.

16. Image the blot using film or an appropriate imaging system.

*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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