2

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Fluorescent Western Protocol V.2

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1

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Analysis of proteins using fluorescent immunoblot.

Note:
- The choice of secondary antibody depends on the choice of primary antibody, whether it is derived from a mouse (monoclonal) or a rabbit (polyclonal).
- It is advisable to stick to the 800CW wavelength to avoid problems with chlorophyll autofluorescence encountered with the 680CW antibodies.

Literature:
Licor's "Fluorescent Western Blot Detection"
Licor's "Good Westerns Gone Bad"

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Lynn Doran

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Adjusted blocking and wash buffer components. Added notes with tips and tricks.

Western Blot, Fluorescent Western Protocol, Protein Analysis

protocol ,

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Reagents

- Intercept^{R} PBS Blocking Buffer (LI-COR Biosciences; 927-70001)
  Intercept® T20 (PBS) Antibody
- Diluent **Licor Catalog #927-75001** In 2 steps
- IRDye® 800CW Donkey anti-Rabbit IgG Secondary Antibody (LI-COR Biosciences; 926-32213)
- IRDye® 800CW Donkey anti-Mouse IgG Secondary Antibody (LI-COR Biosciences; 926-32212)
- Black Western Blot Incubation Box (LI-COR Biosciences; 929-97110)
- 10x PBS buffer, Lonza™ BioWhittaker™ Phosphate Buffered Saline (10X) (Fisher Scientific;BW17-517Q)
- Tween^{TM} 20 (Fisher Biosciences; BP337-100)
- Instant Nonfat Dry Milk
- Methanol
- Primary antibody (various)

Materials

- Graduated cylinder
- Reagent bottle
- Black western blot incubation box (LI-COR Biosciences; 929-97301)
- Micropipette, 1000 ul
- Micropipette, 10 ul
- Pipette tips, 100-1000 ul
- Pipette tips, 1-10 ul

Equipment

- Odyssey CLx Imager (LI-COR Biosciences)
- Shaking platform

Isolate total protein via Leaf Protein Extraction for Immunoblot (Soybean, Cowpea, Tobacco).
Quantify protein via Protein Concentration Determination using Qubit 4 Fluorometer.
Separate protein components via SDS-PAGE gel electrophoresis.
Transfer protein to a membrane via Protein Transfer using Bio-rad TransBlot Turbo.

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1

Keep membranes in the black Western Blot incubation box for all steps, this is important after adding the secondary antibody because the signal is light-sensitive and will become bleached if not exposed to light for a long enough period.

Boxes should be thoroughly cleaned between uses. Residual protein contamination from previous blots can lead to high background signal. If background signal is increasing, clean boxes with 70% ethanol and dry thoroughly between uses.

2 Wet with 1x PBS for **00:02:00** min

2m

3 Rinse membrane with dH_2O

4 Discard PBS and incubate with Intercept (PBS) Blocking Buffer **LI-COR Catalog #927-70001** **01:00:00** at **Room temperature**

1h

Blocking prevents unspecific binding of antibody and lowers background signal.

As an alternative to Intercept Blocking Buffer you can use PBS 5% w/v milk powder (no Tween-20). Typical mini-blots use ~15 mL blocking buffer (15 mL 1X PBS, 0.75 g non-fat dry milk). Do not use milk powder as a blocking agent if protein of interest is phosphorylated.

5 Prepare **1 L** PBS-T solution by diluting 100 mL of 10X PBS

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6 Incubate with primary antibody (appropriate dilution in
Intercept® T20 (PBS) Antibody
Diluent **Licor Catalog #927-75001** at **4 °C**
Overnight (in cold room) with gentle agitation on a platform shaker **50 rpm, 4°C** .

As an alternative to Intercept Antibody Diluent you can use PBST (0.1% Tween-20, 5% milk). Typical mini-blots use ~15 mL blocking buffer (15 mL 1X PBST, 0.75 g non-fat dry milk).

1:2,000 to 1:5,000 are common dilutions for primary antibodies. The ideal dilution for the primary antibody will vary based on sample type and antibody binding capacity and must be determined empirically.

7 Pour off the primary antibody and rinse the membrane with PBS-T.

Some primary antibodies can be re-used multiple times depending on the concentration used, in this instance collect the primary antibody in a tube and store the solution at -20 ^{o}C before re-use.

8 Cover the membrane with PBS-T, shake vigorously on a platform shaker at **50 rpm, Room temperature , 00:10:00** . Repeat 3 times.

If high background signal is observed, increase wash time to 20 minutes.

9 Create a working dilution of secondary antibody using
Intercept® T20 (PBS) Antibody
Diluent **Licor Catalog #927-75001** . For PVDF membranes only, add 0.01% SDS to the antibody diluent. Microcentrifuge secondary antibody and pipette from supernatant to precipitate out any protein complexes that may have formed during storage.

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As an alternative to Intercept Antibody Diluent you can use PBST (0.1% Tween-20, 5% milk). Typical mini-blots use ~15 mL blocking buffer (15 mL 1X PBST, 0.75 g non-fat dry milk).

1:20,000 is a common dilution for secondary antibodies. Consult manufacturer's recommendations and the ideal dilution for the secondary antibody will vary based on sample type and antibody binding capacity and may need to be determined empirically.

10 Incubate for **01:00:00** **Room temperature** with gentle agitation on a platform shaker. 1h

Incubation longer than one hour will lead to high background signal.

11 Pour off the secondary antibody and rinse membrane with distilled water to remove residual blocking agent.

12 Cover the membrane with PBS-T, agitate **80 rpm, Room temperature , 00:10:00**

If high background signal is observed, increase wash time to 20 minutes.

13 Discard PBS-T. Repeat step 12 three times.

More washes (x5) and for longer can be done to reduce background

14 Rinse then cover the membrane with 1x PBS, **80 rpm, Room temperature , 00:10:00** .

15 Proceed to imaging blot on LI-COR Odyssey CLx imaging system

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Odyssey CLx

Imaging System

LI-COR **Odyssey CLx**

Ensure that the platform of the Odyssey CLx is thoroughly cleaned of residual protein from previous blots using isopropanol.

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