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Fluorescent Western Protocol V.2

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Protocol status: Working

We use this protocol and it's working

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Abstract

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"](#)

Materials

Reagents

- Intercept^R PBS Blocking Buffer (LI-COR Biosciences; [927-70001](#))
-  Intercept[®] T20 (PBS) Antibody Diluent **LI-COR Catalog #927-75001**
- 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[™] 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

Protocol materials

 Intercept[®] T20 (PBS) Antibody Diluent **LI-COR Catalog #927-75001**

 Intercept (PBS) Blocking Buffer **LI-COR Catalog #927-70001**

 Intercept[®] T20 (PBS) Antibody Diluent **LI-COR Catalog #927-75001**

 Intercept[®] T20 (PBS) Antibody Diluent **LI-COR Catalog #927-75001**

Troubleshooting

Before start

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](#).

- 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.



Note

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 dH₂O

- 4 Discard PBS and incubate with

 Intercept (PBS) Blocking Buffer **LI-COR Catalog #927-70001**  01:00:00 at

 Room temperature

1h

Note


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

- 6 Incubate with primary antibody (appropriate dilution in

 Intercept® T20 (PBS) Antibody Diluent **LI-COR Catalog #927-75001** at  4 °C

 Overnight (in cold room) with gentle agitation on a platform shaker

 50 rpm, 4°C .

Note

As an alternative to Intercept Antibody Diluent you can use PBST (0.1% Tween-20, 5% milk). Typical mini-blot 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.

Note

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 °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.

Note

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 **LI-COR 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.

Note

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

Note

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

Note

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

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

Note

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

Equipment

Odyssey CLx	NAME
Imaging System	TYPE
LI-COR	BRAND
Odyssey CLx	SKU
https://www.licor.com/bio/odyssey-clx/	LINK



Note

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