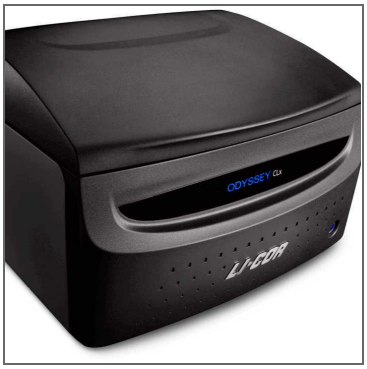

VERSION 2

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OPEN ACCESS

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**Protocol status:** Working
We use this protocol and it's working

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# Fluorescent Western Protocol V.2

Version 1 is forked from Fluorescent Western Protocol

Steven J
Lynn Doran[1], Burgess[2]

[1]Realizing Increased Photosynthetic Efficiency (RIPE);
[2]University of Illinois at Urbana-Champaign

UIUC Long Lab

Lynn Doran
Realizing Increased Photosynthetic Efficiency (RIPE)

## ABSTRACT

Analysis of proteins using fluorescent immunoblot with optional total protein stain.

**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"

## GUIDELINES

Do not touch the membrane. Always use tweezers to move the membrane to avoid protein contamination from your hands. Always use clean materials to handle and store the membranes as residual protein from dirty materials can cause high background on the membranes.

**PROTOCOL integer ID:**
87732

**Keywords:** Western Blot, Fluorescent Western Protocol, Protein Analysis

## MATERIALS

### Reagents

- Intercept$^{R}$ PBS Blocking Buffer (LI-COR Biosciences; 927-70001)
- Intercept® T20 (PBS) Antibody Diluent **Licor 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$^{TM}$ 20 (Fisher Biosciences; BP337-100)
- Instant Nonfat Dry Milk
- Methanol
- Primary antibody (various)
- Ultrapure water.
- Optional: Revert™ 700 Total Protein Stain and Wash Solution Kit (Licor 926-11015)

### 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
- Incubator, capable of 37C.

## PROTOCOL MATERIALS

Intercept (PBS) Blocking Buffer **LI-COR Catalog #927-70001** Step 18

Intercept® T20 (PBS) Antibody Diluent **LI-COR Catalog #927-75001** Step 20

BEFORE START INSTRUCTIONS

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 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.

## Optional: Revert 700 Total Protein Stain

2h 2m

2 Add methanol to the Revert 700 Total Protein Stain reagents as indicated on each bottle.

3 Rinse the transfer buffer from the membrane after transblot using ultrapure water.

4 Place the membrane on a Whatman 1 filter paper or drape it across the seam of the incubation box and dry at 37C for 10 minutes.

Alternatively, it can be dried overnight at room temperature if short on time on day 1.

5 Rehydrate the membrane for 5 minutes.
- Nitrocellulose: rehydrate in PBS for 5 minutes with shaking.
- PVDF: Rehydrate in 100% methanol for 30 seconds, then in PBS for 5 min with shaking.

6 Rinse membrane with ultrapure water.

7 Stain membrane with 5 mL of Revert 700 Total Protein Stain.

Measure the volume with a graduated cylinder to save expensive reagents.

8 Incubate with shaking for 5 minutes at room temperature.

9 Discard stain in appropriate hazardous waste.

10 Add 5 mL of Revert 700 Wash Solution.
Measure the volume with a graduated cylinder to save expensive reagents.

11 Incubate with shaking 30 seconds at room temperature.

12 Discard wash in appropriate hazardous waste.

13 Repeat 5 mL Revert 700 Wash.

14 Discard wash solution and rinse membrane with ultrapure water.

Note

This optional protocol is for total protein quantification for a single-color western blot on the 800 nm channel only.  If a two-color western blot  on both 700 and 800 nm channel is planned, refer to the Revert 700 Total Protein Stain Insert for the appropriate destain procedure.

15 Image on 700 nm channel on Odyssey Imaging System.

Note

Note: UIUC IGB reserve Odyssey Imaging System at https://www-app.igb.illinois.edu/cabbical/.

## Fluorescent Western Protocol

16 Wet with 1x PBS for 00:02:00 min 2m

17 Rinse membrane with $dH_2O$

18 Discard PBS and incubate with 1h

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

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.

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

20 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-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.

21 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 $^{o}$C before re-use.

22 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.

23 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.

> **Note**
>
> Ensure that secondary antibody is compatible with primary antibody. For plants, an antibody that fluoresces at 800 nm is recommended. Chlorophyll fluoresces near 700 nm and may cause high background signal or interfere with target signal.

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

> **Note**
>
> Incubation longer than one hour can lead to high background signal.

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

**26** 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.

**27** Discard PBS-T. Repeat step 12 three times.

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

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

29 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.