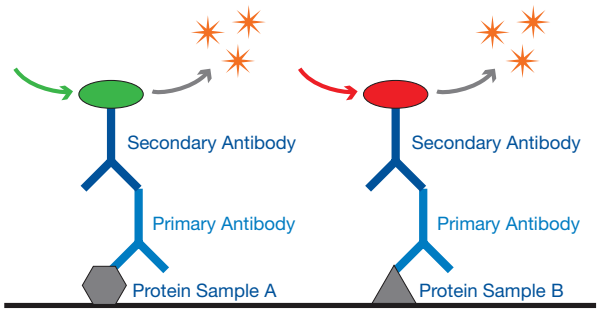
How to Improve Your Fluorescent Western Blots

Fluorescent Western blots are the gold standard for quantitative Westerns. They are ideal for detecting multiple proteins simultaneously (multi-plexing), allowing in-lane normalization and detection of your protein of interest and loading control at the same time. In addition, post-translational modifications can be studied and quantitated easily.

In fluorescent Western blotting, the secondary antibody is directly conjugated to a dye, which is excited by light. The emitted light is detected by a digital imager and digitized for data analysis. Multiple proteins can be detected simultaneously by using secondary antibodies conjugated to different dyes with non-overlapping spectral emissions.



Although similar to chemiluminescent Westerns, there are additional factors that must be taken into consideration when performing fluorescent Westerns:

- Titrate both primary and secondary antibodies. Use a dot blot and checkerboard titration to determine the optimum primary and secondary antibody concentrations.
    - When multi-plexing, optimize detection of each target separately prior to simultaneous detection.
    - Primary antibodies may need to be increased 2-5x compared to concentrations used in chemiluminescent Westerns.
    - Secondary antibodies may also need to be increased (1:5000 is a recommended starting dilution).
- Use a PVDF membrane with low autofluorescence. Nitrocellulose and some PVDF membranes can autofluoresce causing high background.
- Avoid inks and dyes that can fluoresce. Use a pencil to mark the blot. Common dyes such as bromophenol blue and Coomassie autofluoresce.
- Keep everything clean. Prevent background by thoroughly cleaning all equipment and trays prior to use. Only handle the gel and membrane with gloved hands. Use powder-free gloves. Keep trays covered during incubations.
- If you are using fluorescent molecular weight markers, skip a lane before loading samples. This will prevent the signal from the molecular weight markers from bleeding into sample lanes.
- Work with fluorescent antibodies on the bench top, but store stocks in the dark.
- When multiplexing, use primary antibodies made in different species and secondary antibodies that are highly cross-adsorbed to prevent cross recognition.
- Avoid spectral overlap when multiplexing. Choose fluorophores that have optically distinct spectra.
- To increase the specific signal, always detect the strongest target in the blue channel, the middle in the green channel and the weakest in the red channel.
- When archiving blots, store them in the dark.

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