

Transfer and staining of proteins in western blot

Detailed protocol for the transfer and staining of proteins in western blot. Includes visualization of proteins in gels, transfer and development methods.

Contents

- Visualization of proteins in gels
- Transfer
- Visualization of proteins in membrane with Ponceau Red
- Blocking the membrane
- Incubation with primary antibody
- Incubation with secondary antibody
- Development methods

Visualization of proteins in gels

Protein visualization at this stage is useful to determine if proteins have migrated uniformly and evenly. Use the copper stain if you plan to transfer the separated proteins to a membrane, as the Coomassie stain is not reversible. Only use the Coomassie stain on gels post-transfer to check the efficiency of the transfer, or if you have no plans to transfer and just want to observe the results of the SDS-PAGE separation.

Coomassie stain

As soon as the power is turned off the separated protein bands will begin to diffuse (they are freely soluble in aqueous solution). To prevent diffusion of proteins treat the gel with a 40% distilled water, 10% acetic acid, and 50% methanol solution which causes almost all proteins to precipitate (become insoluble).

To visualize the fixed proteins place the gel in the same mixture of water/acetic acid/methanol but with the addition of 0.25% by weight Coomassie Brilliant Blue R-250. Incubate for 4 h to overnight at room temperature on a shaker. Transfer the gel (save the dye mixture; it can be re-used many times) to a mixture of 67.5% distilled water, 7.5% acetic acid, and 25% methanol, place on shaker, and replace with fresh rinse mixture until the excess dye has been removed.

The stain will not bind to the acrylamide, and will wash out (leaving a clear gel). However, it remains strongly bound to the proteins in the gel, and these take on a deep blue color.

Copper stain

Briefly rinse freshly-electrophoresed gels in distilled water (30 sec maximum) and then transfer to a solution of 0.3 M CuCl_2 for 5–15 min. Wash the gels briefly in de-ionized water and view them against a dark-field background. Proteins come up as clear zones in a translucent blue background. Gels may be destained completely by repeated washing in 0.1-0.25 M Tris/0.25 M EDTA pH 8.0. Move the gel to a dish of transfer buffer before proceeding with transfer according to the transfer apparatus manufacturer's instructions.

Transfer

Detailed instructions for the transfer process can be found on the websites of the manufacturers of transfer apparatus, and will vary depending on the system. The principle is the same in each case though. Just as proteins with an electrical charge (provided by the SDS bound to them) can be induced to travel through a gel in an electrical field, so can the proteins be transferred in an electrical field from the gel onto a sturdy support, a membrane that blots the proteins from the gel. Early methods relied on diffusion; blotting in an electrical field is now standard.

Transfer can be done using a wet or semi-dry system. Wet transfer is less prone to failure due to drying of the membrane, and is especially recommended for large proteins. For both kinds of transfer, the membrane is placed next to the gel. The two are sandwiched between absorbent materials, and the sandwich is clamped between solid supports to maintain tight contact between the gel and membrane.

In a wet transfer, the gel and membrane are sandwiched between sponge and paper (sponge > paper > gel > membrane > paper > sponge) and all are clamped tightly together to ensure that no air bubbles form between the gel and the membrane. The sandwich is submerged in transfer buffer to which an electrical field is applied. The negatively-charged proteins travel towards the positively-charged electrode, but are bound by the membrane, preventing them from continuing on.

A standard buffer for wet transfer is the same as the 1x Tris-glycine buffer used as the gel running buffer, but without SDS and with the addition of methanol to a final concentration of 20%. For proteins larger than 100 kD, it is recommended that SDS is included at a final concentration of 0.1%.

In a semi-dry transfer, a sandwich consisting of paper > gel > membrane > paper wetted in transfer buffer is placed directly between positive and negative electrodes (cathode and anode respectively). As for wet transfer, it is important that the membrane is closest to the positive electrode and the gel closest to the negative electrode.

The proportion of Tris and glycine in the transfer buffer is not necessarily the same as for wet transfer; consult the apparatus manufacturer's protocol. A standard recipe is 48 mM Tris, 39 mM glycine, 0.04% SDS, 20% methanol.

Two types of membrane are available: nitrocellulose and PVDF (positively charged nylon). The choice is personal and both work very well. PVDF membranes require careful pre-treatment: cut the membrane to the appropriate size then soak it in methanol for 1–2 min. Incubate the membrane in ice-cold transfer buffer for 5 min. Failure to equilibrate the membrane in ice-cold transfer buffer will cause shrinking while transferring and a distorted pattern of transfer.

Transfer of large and small proteins

The balance of SDS and methanol in the transfer buffer, protein size, and gel percentage can affect transfer efficiency. The following modifications will encourage efficient transfer:

Large proteins (>100 kD)

- For large proteins, transfer out of the gel may be very slow, just as they run slowly within the gel during separation. If blotting a large protein, be sure to run your samples in a low-concentration gel, 8% or less. These will be very fragile, so handle carefully.
- Large proteins will tend to precipitate in the gel, hindering transfer. Adding SDS to a final concentration of 0.1% in the transfer buffer will discourage this. Methanol tends to remove SDS from proteins, so reducing the methanol percentage to 10% or less will also guard against precipitation.
- Lowering the methanol percentage in the transfer buffer also promotes swelling of the gel, allowing large proteins to transfer more easily.
- Methanol is only necessary if using nitrocellulose. If using PVDF, methanol can be removed from the transfer buffer altogether, and is only needed to activate the PVDF before assembling the gel/membrane sandwich.
- Choose wet transfer overnight at 4°C instead of semi-dry transfer.

Small proteins (<100 kD)

- All proteins are hindered from binding to membranes by SDS but small proteins more so than large proteins. If your protein of interest is small, omit SDS from transfer buffer.
- Keep the methanol concentration at 20%.

The following reference discusses a gel and buffer system that allows transfer of proteins as large as 500 kD:

Bolt MW and Mahoney PA (1997). High-efficiency blotting of proteins of diverse sizes following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal Biochem*, 247, 185–92.

More transfer tips:

- Avoid touching the membrane with your fingers; use tweezers instead. Oils and proteins on fingers will block efficient transfer and create dirty blots.
- After sandwiching the gel and membrane between paper, air bubbles between the gel and membrane can be removed by rolling them out with a roller, pipette or 15 mL tube, or by assembling the sandwich in a dish of transfer buffer to prevent formation of bubbles in the first place.
- Make sure the paper and membrane are cut to the same size as the gel. Large overhangs may prevent a current from passing through the membrane in semi-dry transfers.
- Chicken antibodies tend to bind PVDF and other nylon-based membranes, leading to high background. Switching to a nitrocellulose membrane should help reduce background staining.

Visualization of proteins in membrane with Ponceau Red

To check for success of transfer, wash the membrane in TBST. Dilute the stock Ponceau Red 1:100. The stock is made of 2% Ponceau S in 30% trichloroacetic acid and 30% sulfosalicylic acid.

Incubate on an agitator for 5 min then wash extensively in water until the water is clear and the protein bands are well-defined. The membrane may be destained completely by repeated washing in TBST or water. When using a PVDF membrane, re-activate the membrane with methanol then wash again in TBST.

TBS 10x

For 1 L:

24.23 g Trizma HCl

80.06 g NaCl

Dissolve in 800 mL distilled water

pH to 7.6 with HCl

Top up to 1 L

TBST

For 1 L:

100 mL TBS 10x

900 mL distilled water

1 mL Tween 20

Tween 20 is very viscous and will stick to the tip of your measuring pipettes. Be sure you add the right amount of the detergent to the Tris buffer. A 10% solution is easier to dispense than undiluted Tween 20.

Blocking the membrane

Blocking the membrane prevents non-specific background binding of the primary and/or secondary antibodies to the membrane (which has a high capacity for binding proteins and therefore antibodies).

Two blocking solutions are traditionally used: non-fat milk or BSA (Cohn fraction V). Milk is cheaper but is not recommended for studies of phosphor-proteins; milk contains casein which is a phospho-protein, causing high background because the phosphor-specific antibody detects the casein present in the milk.

Some antibodies give a stronger signal on membranes blocked with BSA as opposed to milk for unknown reasons. Check the application notes on the datasheet in case there are specific instructions on how to block the membrane.

To prepare a 5% milk or BSA solution, weigh 5 g per 100 mL TBS with Tween 20 (TBST) buffer. Mix well and filter. Failure to filter can lead to spotting where tiny dark grains will contaminate the blot during development.

Incubate for 1 h at 4°C under agitation. Rinse for 5 s in TBST after the incubation.

Incubation with primary antibody

Incubation buffer

Dilute the antibody in TBST at the suggested dilution. If the datasheet does not have a recommended dilution try a range of dilutions (1:100–1:3000) and optimize the dilution according to the results. Too much antibody will result in non-specific bands.

It is traditional in certain laboratories to incubate in blocking buffer, while other laboratories incubate the antibody in TBST without a blocking agent. The results are variable from antibody to antibody and you may find it makes a difference to either use a non-blocking antibody buffer or the same agent as the blocking buffer.

If high background is not an issue, some antibodies produce a much stronger signal if diluted in buffer with low concentrations (0.5–0.25%) of milk or BSA, or none at all.

Incubation time

The time can vary between a few hours to overnight (rarely more than 18 h), and is dependent on the binding affinity of the antibody for the protein and the abundance of protein. We recommend a more dilute antibody and a prolonged incubation time to ensure specific binding.

Incubation temperature

Preferably cold. If incubating in blocking buffer overnight, it is imperative to incubate at 4°C or contamination will occur and thus destruction of the protein (especially phosphor groups).

Agitation of the antibody is recommended to enable adequate homogenous covering of the membrane and prevent uneven binding.

Incubation with secondary antibody

Wash the membrane several times in TBST while agitating, 5 min or more per wash, to remove residual primary antibody.

Incubation buffer and dilution

Dilute the antibody in TBST at the suggested dilution. If the datasheet does not have a recommended dilution, try a range of dilutions (1:1,000–1:20,000) and optimize the dilution according to the results. Too much antibody will result in non-specific bands. You may incubate the secondary antibody in blocking buffer, but a reduction in background may come at the cost of a weaker specific signal, presumably because the blocking protein hinders binding of the antibody to the target protein.

Incubation time and temperature

1–2 h at room temperature with agitation.

Which conjugate?

We recommend horseradish peroxidase (HRP)-conjugated secondary antibodies. Alkaline phosphatase (ALP)-conjugated secondary antibodies are less sensitive and are not recommended.

Development methods

Detection kits

For HRP-conjugated antibodies enhanced chemiluminescence (ECL) kits are traditionally used as substrates. We offer HRP substrates with varying detection limits.

X-ray films

Automated x-ray film developers are widely used and easy to use. Remember that an over-exposed film is not suitable for analysis as determination of the relative amount of protein is not possible. Overexposed films show totally black bands with no contrast, and/or numerous non-specific bands.

Digital images

The new generation of film developers are units with a camera inside an enclosure, removing the need for a darkroom. The camera detects the chemiluminescence emanating from the membrane, transforming the signal into a digital image for rapid analysis with software provided with the detection machine.

A range of machines are now commercially available. At the front of the next generation are systems which do not use HRP-conjugated antibodies (i.e. chemiluminescence). For example, STORM Analyzers detect fluorescence from fluorochrome-conjugated secondary antibodies. The Odyssey Infrared Imaging System detects infrared fluorescence.