Nordic BioSite - in Life Science Research

Western Blot Protocols

1. Transfer proteins from gel to membrane (nitrocellulose of PVDF): Gloves should be worn when handling gels or blot membranes.

A. Pour enough transfer buffer (or CAPS buffer) into a container so that half of the assembled cassette will be 0.5cm below the surface of the buffer.
B. After the gel has finished running, carefully remove the front glass plate and remove the stacking gel. Remove a small portion of the upper left-hand corner of the running gel to mark its orientation during the transfer process.
C. Wet the membrane with transfer buffer and carefully lay it across the exposed running gel, making sure that air nubbles between the gel and membrane are excluded. In the same fashion, add two layers of pre-wetted filter paper to the membrane. Pre-wet one of the cassette sponges and add to the stack. Top with the outer half of the cassette itself and carefully turn the entire stack of glass plate, gel, membrane, filter paper, sponge and cassette over. Place the stack in the container with transfer buffer.
D. Carefully remove the second glass plate from the gel. Using the same process, make sure that no air bubbles have formed between the gel and the membrane.
E. Keeping the stack generously wet, top the gel with two layers of pre-wet filter paper, wet sponge and the remaining half of the cassette unit. Snap the cassette unit together firmly.
F. Place the cassette into the transfer unit so that the current will flow through the gel onto the membrane. This would be with the gel closer to the black (-) cathode and the membrane closer to the red (+) anode.
G. Add transfer (or CAPS) buffer to the transfer unit according to manufacturer's directions. Transfer over night with a setting of 20V / 40 mA or for 3 hours at 70V/160 mA. The transfer process needs to take place under cold temperatures to prevent the gel from sticking to the membrane. This can be accomplished either by using a water cooling core in the transfer unit or placing the entire unit at 4°C. Please follow the manufacturer's recommendations.
H. At the end of the transfer period, disconnect the power connections and disassemble the cassette. All of the Coomassie Blue-dyed bands should now be on the membrane leaving the gel fairly clear.

2. Staining the Blot (Optional):

A. Briefly with water, rinse any bits of gel off the blot then incubate it in ponceau S solution for 1 minute. Rinse in water until the background is reduced.
B. Place the membrane on a transparency sheet and photocopy the blot for a permanent transfer record. C. Rinse the blot several times in TBS-Tween to remove the stain.

3. Blocking the Blot:

A. Gently shake the blot for 2 hours in 5% non-fat dry milk/TBS/Tween at room temperature or at 4°C on a rocker.
B. Either pour off blocking buffer or transfer the blot to a sealing bag for probing.

4. Probing the Blot:

A. Dilute the primary antibody in 5% non-fat dry milk/TBS. Add to the blot, seal the container and gently rock for 2 hours at room temperature or over night at 4° C. If using a bag for the incubation, eliminate all air bubbles before sealing.
B. Remove the primary antibody and wash the blot 3 x 5 minutes in TBS/Tween. In some instances, the primary antibody can be saved for additional blot procedures.
C. Dilute the secondary antibody in 5% non-fat dry milk/TBS. Add to the blot, seal and incubate as before.
D. Remove the secondary antibody and wash the blot 3 x 10 minutes in TBS/Tween.

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5. Detection / Chemiluminescence:

A. Cut two transparency sheets to fit into the film cassette.
B. Mix oxidizing and luminol reagent in a 1:1 ratio.
C. Pour onto a piece of parafilm and lay the blot on the pool of solution for 1 minute.
D. Insert the blot into the cassette and top with the remaining sheet of transparency, making sure that all bubbles are eliminated.
E. Insert film and close cassette. Start with a 1 minute exposure and adjust from there. The signal will decay over time. If no bands are found, the blot can be rinsed and stored in PBS/Tween overnight for repeated blocking and probing.

6. Stripping and Reprobing Blots : Works well with chemiluminescence and radioactive detection, but is not useful in colorimetric detection due to the production of an insoluble reaction product formed as a results of that detection method.

A. Briefly rinse the blot in water.
B. Wash the blot 2 x 10 minutes in TBS/Tween.
C. Re-block in 5% milk/TBS/Tween for one hour at room temperature.
D. Add stripping buffer to the blot, seal the container and incubate for 30 minutes at 50° C. Occasionally agitate the container for best results.
E. Wash the blot 2 x 30 minutes followed by 2 x 10 minutes in TBS/Tween.
F. If desired (optional), can incubate the blot in the detection reagent and expose to film to ensure that no residual detection antibody remains. In order to check for the presence of primary antibody, the blot would have to be incubated with secondary antibody and detection reagent to verify complete removal.
G. Block the blot for 2 hours in 5% milk/TBS/Tween at room temperature before probing with the desired primary antibody again.