Ten Tips for Successful Westerns

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1. Avoid Protein Degradation

Samples should always be prepared quickly, cooled on ice and pan-protease and phosphatase inhibitors added to avoid degradation of proteins. Be aware that commonly used protease inhibitors such as phenylmethanesulfonylfluoride (PMSF) may have short half-lives and need to be re-supplemented with time.

2. Prepare Your Samples Appropriately

In general, samples should be boiled five minutes in sample buffer of choice, centrifuged and only the supernatant should be used for SDS-PAGE analysis. If gels are run under denaturing conditions, the actual concentration in reducing agent (e.g. dithiothreitol (DTT) or β-mercaptoethanol) in your sample buffer will need to be high enough to effectively break-up all proteinaceous disulfide bonds. If you are uncertain whether your sample buffer still contains enough reducing agent (NOTE: its effective concentration will decrease with time!), simply add extra. It will do no harm to your samples and you will be on the safe side.

3. Boiling vs. Non-boiling of Samples

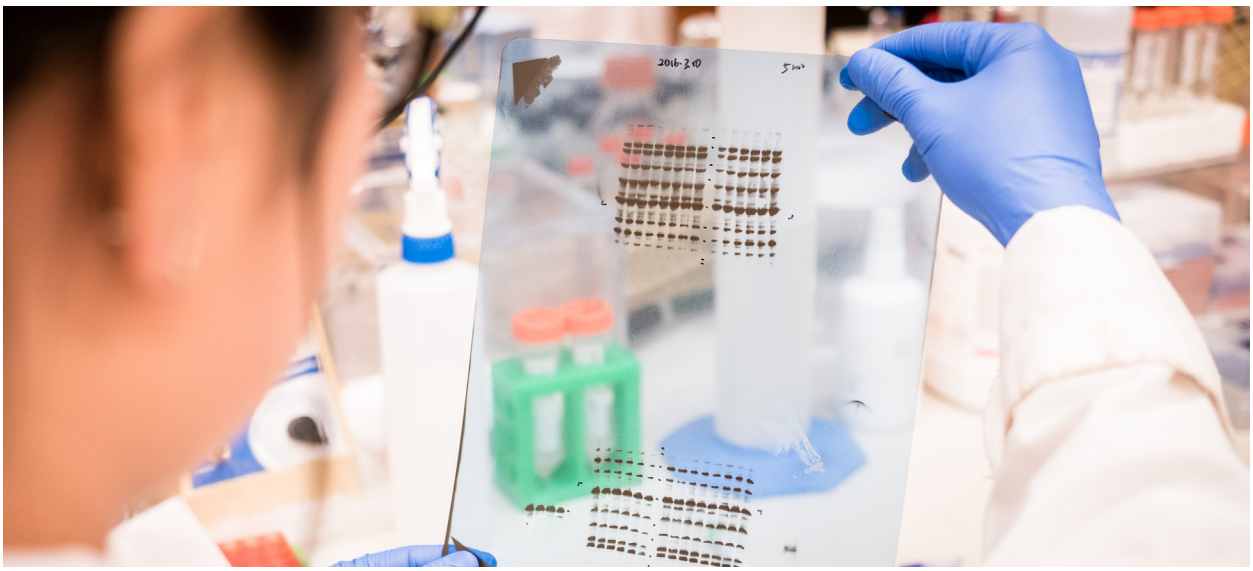
Be aware that highly glycosylated or hydrophobic proteins such as multi-drug resistance (MDR) proteins may precipitate upon boiling. In those instances, you should consider incubating your samples with sample buffer at 60 °C for one hour instead of boiling for five minutes.

4. Denaturing vs. Blue/Native Gels

The standard SDS-PAGE for subsequent Western blot analysis is being done under reducing and denaturing conditions. In some cases, this treatment may, however, disrupt the conformation of a three-dimensional epitope that is recognized by a monoclonal antibody. In those instances, a blue/native gel needs to be run. Be aware that due to retaining its three-dimensional structure, your target protein will show a higher apparent molecular weight (MW) in blue/native gels than under standard denaturing conditions.

5. Adapt Gel to Match the Requirements of Your Target Protein

Choose the type of gel and its polyacrylamide (PA) content depending on the MW of your target protein and the required accuracy of the MW determination. Standard SDS-PAGE gels range from 7 to 15% PA. To get a good resolution, lower concentrations of PA should be used as the MW of target proteins get higher. Especially for complex protein mixtures that are spanning a high MW range, you may need to deploy gradient gels for best resolution. Note that the standard SDS-PAGE using Laemmli glycine-based buffers has a minimum resolution of approximately 16kDa. Hence, for smaller proteins and peptides, alternative methods such as the one developed by Schägger and von Jagow (1987) may be necessary.



6. Transfer of High MW Proteins

Please note that high MW proteins (above 100kDa) generally transfer more efficiently overnight in a wet transfer apparatus with the addition of SDS in the transfer buffer. Also, it may be necessary to eliminate methanol from the transfer buffer as methanol inhibits elution of high MW proteins out of the gel matrix. It is recommended to use a PVDF membrane instead of nitrocellulose for high MW proteins. Also, use Ponceau S to make sure proteins were transferred from the gel efficiently onto the membrane and stain the gel with Coomassie Brilliant Blue.

7. Transfer of Proteins with High Isoelectric Point (pI)

To transfer a protein from gel to membrane, pH of the transfer buffer must be higher than the pI of the respective protein, since only then migration towards the positively charged anode during electrophoresis can occur and transfer to the membrane can happen. For proteins with a pI equal or higher than the pH of the transfer buffer (usually around 8.3), SDS can be included into the transfer buffer to add negative charges to the proteins. In addition, a semi-dry transfer system may need to be used.

8. Do Not Deem an Antibody to be Unspecific Until You Have Efficiently Reduced Background

Be aware that not every antibody and sample type will directly match your established Western blot protocol and give you a nice clean blot at first shot. There are a plethora of parameters that may lead to background staining, which may easily be mixed-up with the unspecific staining of an antibody. By careful assay optimization, you should almost always be able to substantially reduce background and get a nice clear signal in your Western blots. The use of appropriate controls is essential in this optimization process.

9. Check That the Blocking Procedure is Appropriate for Your Target Protein

The standard blocking agent in Western blot is non-fat dry milk. However, performance of some antibodies may be adversely affected by blocking with milk or casein, since the antigens/epitopes they detect may be present in those blocking agents at concentrations that can be high enough to elevate the background of a blot and obscure a positive signal. Therefore, use of milk or casein should always be avoided for, antibodies against Ubiquitin, phosphoserine or phosphothreonine. In those instances, use serum of the secondary antibody host or BSA for blocking, and for incubation with the primary antibody.

10. Always Use Well-established Controls to Validate Your Assays

It is crucial to validate your Western blots with reliable positive and negative controls. Particularly when looking at endogenous proteins produced in low amounts, even a good positive control lysate may require input of up to 150µg of total protein per lane. Also, cells transfected with your target protein or the purified (recombinant) protein may serve as a good source for positive controls. As a negative control, you should always run a "secondary antibody only" blot in parallel to assess how much background actually stems from the secondary antibody and detection system deployed. If available, the immunogenic peptide an antibody was raised against can also be used as a blocking peptide and be very helpful in distinguishing the true signals of your target protein from background staining.

Although these are some tips, there are clearly many other issues that need to be considered and addressed in order to ensure a successful experiment, such as washing buffer, antibody concentration, secondary antibody etc. However, the key thing is to understand the principles and practice of Western blotting, and be mindful of the drawbacks and boundaries of this approach for protein analysis.

Did You Know?

The Southern blot is named after British biologist Edwin Southern. All other blotting methods such as Western blots, and Northern blots, are named in reference to Southern's name.