Tips and tricks of the trade

Crunchy Green Things and Beyond

Quantitative immunoblotting is a great tool to quantify plant proteins – as long as you follow the right protocol.

Lab Hint

Natalie Donaher, plant researcher and quantitative Western blotting expert at the Canadian start-up company Environmental Proteomics, provides ten tips for quantitative Western blots that should work for almost any plant material ranging from diatoms to dicots. Since Environmental Proteomics is a university start-up company created by academics, these are also the methods passed along to students to help them get the most consistent Western Blot results every time.

1. Keep them cold. Samples need to be frozen in liquid nitrogen immediately after harvesting, then stored in a -80 °C freezer until use. Cell cultures can be spun into a pellet or filtered onto binder-free glass fibre filters, before being frozen. Long-term storage of samples at -80 °C appears to reduce protein extraction efficiency, so use them as quickly as possible (and be suspicious of those unlabelled pellets left behind by the last graduate student). We use the filter method when on sampling cruises because the filters catch everything and store well.

2. Grinding plant tissue should be done in a pre-cooled mortar with pestle until a powder can be transferred to the extraction buffer, while filters containing cells are just cut up and submerged. Process the samples in extraction buffer that contains the detergent LDS rather than SDS, since LDS is a less-harsh detergent that doesn't precipitate as easily at lower temperatures. Protease inhibitors (like Pefabloc/AEBSF) should also be present in the buffer but we don't add a reducing agent (like DTT) at this step, since it interacts poorly with the protein assay used downstream.

3. Vertically freeze the sample (either in a micro-centrifuge tube or a 15 mL blue-cap tube) in liquid nitrogen and "soni-thaw" for effective release of proteins. Soni-thaw involves applying the micro-tip of a sonicator (at 30% of maximum output) to the frozen buffer/tissue combination and grinding through the solid mass until it attains a "green slushy" texture. Three soni-thaw cycles should be appropriate for plant tissue but diatoms and cyanobacteria might need a few more rounds. Test the efficacy of the soni-thaw method by micro-centrifuging the results briefly (3 to 5 minutes) – if the supernatant is as dark as or darker than the pellet, you have reached the end of the soni-thaw step. If the supernatant is still a lighter green colour compared to the pellet, keep freezing and sonicating until the supernatant is ready to load onto the gel or store in the freezer.

4. Run a protein assay on the samples (such as the Biorad DC assay, which is detergent compatible) on a microplate to save protein volume. Use a standard in the assay that will react to the dye in a similar way to your protein extract – in many cases that isn't BSA (usually bovine gamma globuline, BGG, works well). While some researchers use chlorophyll or wet tissue weight to guide the consistent loading of gels, we load based on total protein amounts. We can then be consistent between roots, leaves or filters containing environmental samples.

5. If you are trouble-shooting the extraction and electrophoresis process with highly hydrophobic membrane proteins, you can try adding twice as much solubilisation buffer to the sample during extraction. Increasing the buffer from 1-fold to 2-fold during this step will give you more LDS for those hydrophobic proteins and will make the proteins run farther in your acrylamide gel (since your protein will have a greater charge-to-mass ratio than the molecular weight prediction tools calculate). During electrophoresis, the addition of 2-8 M urea in the gels and buffers could help keep your protein unfolded. We often find these types of proteins run as blobs rather than flat bands on our gels.

6. For quantitative immunoblotting, we recommend loading only 1 μg of protein per lane but we always standardise the loading volumes with the addition of a pre-dyed loading buffer and a reducing agent, such as DTT. Although this is a small amount of protein, it allows for quantitative determination of the antibody signal with a good chemiluminescent reagent like ECL Ad-

Looks like preparing a witch's brew, however, freezing plant samples on liquid nitrogen during grinding in a mortar is crucial to get high protein yields. After grinding, the plant powder is transferred to a reaction tube filled with extraction buffer, vertically freezed in liquid nitrogen and finally sonicated (soni-thawed) to release the proteins.

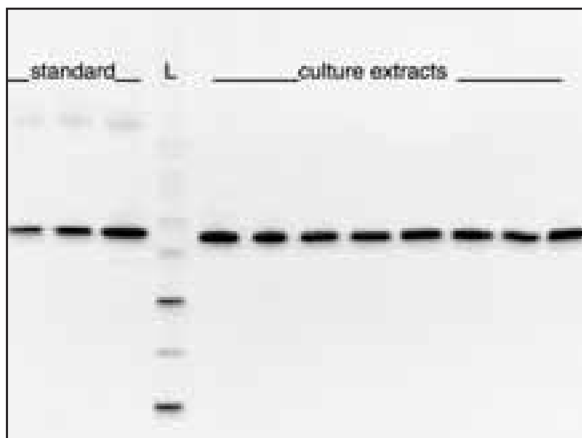
vance (made by GE) combined with a sensitive CCD camera.

7. Run PAGE gels on a consistently performing rig (for example Invitrogen NuPage) – and always use markers and standards on every gel. Cool the running buffer overnight at 4 °C to keep the gel from overheating, and optimise the gel running times for the size of your protein, based on the migration of the pre-stained marker.

8. Ideally, we optimise the quantitative transfer of the protein onto either PVDF or nitrocellulose by adjusting the time used to process the gels. There are a few tricks to apply in troubleshooting this part. For example, if you add a pre-stained marker to every gel, you will be able to see whether the stained ladder transferred to the membrane evenly. Alternatively, if you stain the gel after transfer with Coomassie or something similar, you can tell where the transfer was patchy or the least efficient (usually at the top of the gel where the higher weight proteins are found). Finally, if you fear you are transferring small proteins (<12 kDa) right through your membrane, just put a second membrane behind the first, incubating the second membrane with your antibody to be sure there is no protein present.

9. During incubation with a primary antibody, there must be an excess of antibody for the results to be quantitative. The primary dilutions depend on the protein amount per lane and the imaging protocol. For example, given that our blots normally a) contain between 1 and 5 µg of protein per lane, b) are visualised using Agrisera antibodies and ECL Advance and c) are captured with a CCD camera, we use a default dilution of 1:25,000 for our primary antibody. If this dilution increases or decreases, we modify the amount of secondary antibody as well.

10. Once an image is captured via CCD camera, programmes, such as Biorad's QuantityOne software, will allow the user to highlight bands and assign protein quantities based on a standard curve run on the same gel. Binning the data from a CCD camera will allow you to increase the sensitivity, if that option is available. If there are multiple ways to determine the value for "background signal" (e.g. local versus global background), it is important to know how the programme determines this value and how it should be optimised for your particular blots. A global background subtraction works if you have a uniform background signal, while a patchy background will be better suited to a local background subtraction.



If you follow the ten tips, your Western should look like the image above, showing a quantitative immunoblot of AtpB protein from the freshwater cyanobacteria *Synechocystis*.

Natalie Donaher prepares the electrophoresis chamber to run the extracted plant proteins on a PAGE gel.

For more tips and tricks on quantitative immunoblotting – including a Q&A session with attendees at the Plant 2010 conference held in Montreal this summer – check out the video tutorial available on BioCompare: *www.biocompare.com/Multimedia/Video/665/Watch-video-Western-Blot-Tips-and-Trouble-Shooting.html*. To get specific tips on your Western Blotting experiment using your samples, try live chatting with a scientist at *www.agrisera.com*.

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Do you have any useful tips?
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