Western

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Western Blot Protocol for examining amount of ISPS in poplar leaves (High Salt Protocol for reducing background) (Sharkey lab)

(Adapted from Western Protocol by Amy Wiberley, 2005)

Last modified: July 1, 2008

Materials

1L Running buffer: 50mL 20x NuPAGE MES SDS buffer (Invitrogen)
950mL DI water

1L Transfer buffer: 50mL 20x NuPAGE Transfer buffer (Invitrogen)
849mL DI water (749 if blotting two membranes)
1mL NuPAGE antioxidant (Invitrogen)
100mL methanol (200mL if blotting two membranes)

SDS loading buffer (adapted from Wiberley 2005 PCE 28:898):
1M Tris-HCl, pH 6.8
10% SDS
10% beta-mercaptomethanol
20% glycerol
0.004% bromphenol blue

To make 50mL of SDS loading buffer w/o protease inhibitor:
6.057g Tris
5g SDS
5mL beta-mercaptomethanol
10mL glycerol
Top to 50mL w/ DI water, pH to 6.8
0.002g bromphenol blue

Protease Inhibitor Cocktail for plants (Sigma) – 1mL recommended for inhibition of proteases extracted from 30g of plant tissue in total volume of 100mL)

Add 200uL protease inhibitor to 19.5mL of (SDS loading buffer w/o inhibitor) to make 20mL of loading buffer.

Protein standard

Dilute ISPS to twice the desired concentration, and mix with SDS loading buffer in a 1:1 ratio. (e.g. to make a 1000x dilution, mix 1 part of ISPS stock with 499 parts of DI water and 500 parts of SDS loading buffer)

Ladder

Mix protein ladder stock with SDS loading buffer in a 1:19 ratio.

Gel: NuPAGE 4-12% Bis-Tris 1.0mmx15 well NP0323BOX (Invitrogen)

2′ Ab: ECL anti-rabbit IgG HRP-linked whole antibody (from donkey) (GE Healthcare)

Filter papers cut into 8.5cmx8.5cm pieces.
NuPAGE mini-cell
NuPAGE blot module
Power supply
Blotting pads
A large lasagna dish
~10cm x 20cm tupperwares
GE Healthcare Amersham/Millipore Immobilon PVDF membrane (nitrocellulose membrane

okay – need optimization)
10x TBS: 24.228 Tris, pH to 7.5 w/HCl; 292.2g NaCl; H2O to 1L
5x HST: 12.114 Tris, pH to 7.5 w/HCl; 292.2g NaCl; H2O to 1L
TBS: 100mL 10x TBS; 900mL H2O
TBS-T: 100mL 10x TBS; 899mL H2O; 1mL Tween-20
HST: 200mL 5x HST; 795mL H2O; 5mL Tween-20
TBS-3%BSA: 6g BSA in 200mL TBS (remake every month)
HST-3%BSA: 15g BSA in 500mL HST (remake every month)

Running buffer and transfer buffers can be re-used many times.

I. Crude extract preparation

Method 1: Make 10mm-diameter circular leaf samples with the leaf punch. Ground the leaf sample with mortar and pestle in liquid nitrogen and then add SDS loading buffer to the mortar. Collect the liquid sample in the mortar.
(Very thorough breakage of solid material; 1/3-1/2 of liquid volume is usually lost and cannot retrieved)

Method 2: Make 10mm-diameter circular leaf samples with the leaf punch. Ground the leaf samples in 1.5mL tubes in lead blocks immersed in liq.N2 with blue bits. (all 200uL retrieved. Sample breakdown may not be very thorough)

Freeze the samples in -20.

I. SDS-PAGE

1. Heat leaf samples + standard + protein ladder in 70 degrees water bath for 10 minutes.
2. Prepare the precast gels – remove the tape and comb and rinse with distilled water.
   - Make sure there's no air bubbles in the lanes. If there is, flush the gel under dH2O until it's gone or remove with pipette tips.
   - Wash gently so that the wells don't move.
3. Assemble the chamber and fill the upper chamber with 1x running buffer. Wait a few seconds to make sure there is no leakage into the lower chamber, and then fill the lower chamber with 1x running buffer to at least 1/2 of chamber volume (for heat dissipation).
4. Add 500uL NuPAGE antioxidant to the upper chamber.
5. Load the lanes with ladder, standard and then samples from right to left (front gel).Turn the chambers around and load the other gel from right to left. Close the lid and run under 120V until the blue front has almost reached the bottom (about 75 minutes in MES running buffer).
6. Recycle SDS running buffer, wash mini-cell and its components.

I. Blotting

7. Cut one or two 7.5cm x 8.5cm pieces of PVDF membrane and wet in methanol for 10 seconds (hydration process). Briefly rinse under distilled water and soak in DI water for 5 minutes. Soak in 50mL transfer buffer for 10 minutes. Fold a corner of the membrane to mark the protein side of the membrane, orientation of the membrane and which membrane it is (if blotting two membranes).
8. Soak 6 blotting pads in 1x transfer buffer (~600mL) in a lasagna dish. Press out air bubbles. Soak the 2 (4 if blotting two membranes) filter paper pieces in transfer buffer.
9. Crack open the gel cassette(s) and leave the gel(s) resting on the larger plates. Use the gel knife to cut at 5mm below the wells and remove the gel above this line. Place 1 piece of pre-soaked filter paper on top of each gel, leaving the "foot" of the gel uncovered. Gently press out air bubbles if any.

10. Turn the larger plate(s) over onto the smaller plate(s). Use the gel knife to push to the foot out of the gel cassette and the gel will fall off the plate.
11. Cut off the gel foot. Wet the gel surface with transfer buffer and put the membrane on the gel with "protein side" facing down, gently smoothing out air bubbles if any. Put another piece of pre-soaked filter paper on top of the membrane and press out air bubbles.
12. For one gel: put two soaked blotting pads in the cathode (-) core (the deeper core) of the blot module. Pick up the gel/membrane assembly and put it on the pads so the gel is closest to the cathode plate. Add 4 soaked blotting pads and put the anode (+) core on top.
    For two gels: put two soaked blotting pads in the cathode (-) core, with the first gel/membrane assembly on top (gel closest to the cathode). Add another blotting pad and then the second gel/membrane assembly (gel closest to the cathode). Put 3 more cathode pads and then the anode (+) core.
13. Hold the blot module together firmly and slide it into the guide rails of the lower buffer chamber.
14. Put in the gel tension wedges and make sure they fit tightly.
15. Fill the blot module with transfer buffer just until the gels/membranes are covered, and make sure that it doesn't leak. Fill 3/4 of the outer buffer with 1x transfer buffer.
16. Put on the lid and run the blot at 30V for 1 hour.
17. Take out the membrane and air dry for 2 hours.

I. Immunodetection

All incubating and washing steps should be done in a rectangular Tupperware on rocking shaker at room temperature.

18. Wet in MeOH and then rinse with DI H2O.
19. Incubate the membrane in TBS-3% BSA for 1 hour.
20. Rinse in TBS-T for 5 minutes.
21. Make up 1°Ab solution by dissolving 10uL 1°Ab in 25mL HST-3% BSA. Incubate the membrane in 1°Ab solution for 1 hour.
22. Briefly rinse with DI H2O.
23. Rinse twice in TBS-T, 5 minutes each.
24. Rinse in HST for 5 minutes.
25. Rinse twice in TBS-T, 5 minutes each.
26. Make up 2°Ab solution by dissolving 1uL 2°Ab in 25 mL HST-3% BST. Incubate the membrane in 2°Ab solution for 1 hour.
27. Rinse three times in TBS-T, 5 minutes each.
28. Rinse in HST for 10 minutes.
29. Rinse three times in TBS-T, 5 minutes each.
30. Rinse in TBS for 5 minutes.
31. Make a 1:1 mixture of chemiluminescent enhancer solution and peroxide solution. Place the membrane in a square petri dish and pour the substrate onto the membrane (~12mL/membrane). Put on orbital shaker and shake for 7 minutes. Make sure that the entire membrane is covered with substrate during incubation.
32. Place membrane, protein side down, on a piece of Saran wrap. Strip off all the air bubbles off the edge of bench and fold up the Saran wrap in a way that there's only 1 layer of Saran wrap on the protein side.
33. Autoradiography: (In dark room) Place the wrapped blots, protein side up, in an X-ray film cassette. Place a sheet of autoradiography film on top. Close thee cassette and expose for 15secs – 20mins depending on band intensity (1 minute is usually good). Develop the film manually or on a machine.
    Obtain the image on an imager (LAS-2000): Tape the wrapped blots down a white background (e.g. cardboard) and place in the imager. Select chemiluminescence mode and adjust area and focus. Expose the blot for a desired length of time.
    The luminescent signal is good for one hour and then starts to fade off.

I. Quantification

Autoradiography: scan the film and quantify bands with Adobe photoshop.
Images from Imager: quantify bands with MultiGauge V3.0

Note: for some reason band intensity is inversely correlated with the amount of protein loaded after a first immunodetection. Detect the membrane again without stripping gives a positive correlation.

Other useful protocols (Adopted from Amy′s procotol unless otherwise noted)

Re-wetting membranes if they dried out:
Wet a few seconds in MeOH
Rinse in 2x in dH2O
5min in TBS
15min in TBS-T
15min in HST
(Millipore) Wet with MeOH

Manual development of a Kodak film:

34. Holding the film with metal film hangers, soak the film in a tray of Kodak GBX Developer and Replenisher for 3 minutes with agitation.
35. Remove the film from the developer and place it in a tray of water. Soak with continuous, moderate agitation for 30 seconds.
36. Remove the film from the water and soak it in a tray of Kodak GBX Fixer and Replenisher for 1 minute, with moderate agitation.
37. Remove the film from the fixer and wash it in fresh water for 2 minutes.
38. Remove the film from the water wash and hang it at room temperature until it is dry.

Reprobing the membrane: wash it twice in large volumes of TBS-T, 10 minutes each time, then repeat detection protocol.

Stripping the membrane before reprobing: submerge it in stripping buffer (100mM β-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH6.7) and incubate at 50°C for 30 minutes with occasional agitation. Then wash twice, 10 minutes each time, in large volumes of TBS. Repeat detection protocol.

Coomassie Blue Staining Protocol (Sharkey lab)
(Adopted from Coligan et al., 1995. Current Protocols in Protein Science)

Materials

Coomassie Blue Stain: 0.025% (w/v) Coomassie brilliant blue R-250 (Bio-rad) in 40% methanol/7% acetic acid (v/v)
Destain: 50% methanol/7% acetic acid (v/v)

39. Place membrane in a clear plastic box and wash with DI water three times for 5min each (on orbital shaker 75rpm)
40. Stain membrane with Coomassie blue stain for 5 min (on orbital shaker 75rpm)
41. Destain membrane with 50% methanol/7% acetic acid for 5 to 10 mins (on orbital shaker 75rpm).
42. Rinse with DI water several times and air dry.

Notes:

43. After the transfer is complete, PVDF membranes should be completely dried before continuing onto staining or immunodetection procedures. Drying enhances the adsorption of the proteins to the PVDF polymer. PVDF membranes can be stored dry for up to 2 weeks at 4, up to 2 months at -20, for longer periods at -80.
44. Detection with labeled primary antibody gives weaker signal since the amplifying effect of secondary antibodies are eliminated.
45. Antibodies are diluted in buffer and blocking solution to prevent nonspecific binding to the membrane. The antibody diluent also normally contains trace amounts of Tween-20 or another detergent to prevent nonspecific aggregation of the antibodies.
46. Generall, nonspecific signal can be alleviated by higher dilution of the primary antibody or decreased protein load on the original gel. High overall background can be minimized by higher dilution of the secondary, enzyme-conjugated antibody.