Western Blotting

All steps are carried out at room temperature unless otherwise indicated. Recipes for all solutions are listed at the end of the protocol.

SDS-PAGE

1. Construct an SDS-PAGE gel according to the molecular weight (MW) of your target protein(s). (Recommendations and gel recipes are presented following this protocol).

Tip 1: Tris-tricine gels separate low MW proteins (<20 kDa) better than Tris-glycine gels.

Tip 2: Gradient polyacrylamide gels can provide sharper bands and they separate a broader range of MW sizes on one gel, such as 10-500 kDa.

2. Prepare samples in microfuge tubes. Add 4 X SDS sample buff er so the total protein amount is 30-50 µg per sample (according to the protein amount measured by Bradford or BCA protein assay).

3. Flick microfuge tubes to mix samples, and then heat to 95-100°C for 5 minutes.

4. Set up electrophoresis apparatus and immerse in 1 × running buff er. Remove gel combs and cleanse wells of any residual stacking gel.

5. Load samples and protein markers onto the gel using gel loading tips. Set electrophoresis power pack to 80V (through the stacking gel), before increasing it to 120V when the protein front reaches the separation gel.

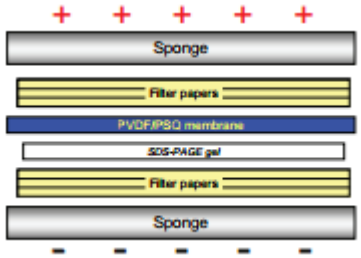
Tip 3: Load generous volumes of sample for the fi rst experiment and adjust as necessary after assessing the initial target signal.

Protein Transfer

6. PVDF membranes (or PSQ membranes with 0.22 µm micropores when MW of target is <30 kDa) are strongly recommended. Soak membranes in methanol for 30 seconds before moving to transfer buffer.

Soak the fi lter papers and sponges in transfer buff er as well.

7. Sequentially assemble the transfer constituents according to the illustration (below) and ensure no bubbles lie between any of the layers. Apply semi-dry or wet transfer systems according to the manufacturer's instructions.



Left: a representation of the components of a transfer "sandwich". Notice the all-important orientation of the gel and membrane; of the two, make sure the PVDF membrane is situated nearest to the positive electrode. Amino acids are negatively charged and proteins will migrate in the direction of the positive charge.

Tip 4: If target MW is larger than 100kDa, wet transfer at 4°C overnight is suggested in place of a semi-dry method; moreover, addition of 0.1% SDS to the wet transfer buff er to facilitate transfer is preferable.

Tip 5: use a 2ml stripette as a "rolling pin", rolling it over the transfer "sandwich" to "iron" out any bubbles that may have been introduced accidentally.

Immunoblotting

8. After transfer, wash the membrane twice with distilled water, and using a pencil, mark bands of the MW ladder on the membrane. If desired, stain the membrane with commercial Ponceau red solution for 1 min to visualize protein bands, then wash any Ponceau red staining with copious amounts of 1X TBST.

9. Block with 1X TBST containing (2-5%) nonfat dry milk (or 1-5% BSA for the detection of phospho-epitope antibodies) with constant rocking for 1 hour or overnight at 4°C.

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10. Dilute primary antibody in blocking solution with a starting dilution ratio of 1:1000. (Optimal dilutions should be determined experimentally.) Incubate the membrane with primary antibody for 1 hour, or overnight at 4°C, on a bench-top rocker.

11. Wash membrane three times with 1X TBST for 10 minutes each.

12. Incubate the membrane with a suitable HRP-conjugated secondary antibody (recognizing the host species of the primary antibody), diluted at 1:5000-1:50000 in blocking solution. Incubate for 1 hour with constant rocking.

13. Wash membrane three times with 1X TBST for 10 minutes each.

Tip 5: Do not let the membrane dry at any stage of the blotting process.

Tip 6: For preservation of the primary antibody solution over long incubations, 0.2% NaN3 could be included in the antibody dilution buffer.

Signal detection

14. Prepare ECL substrate according to the manufacturer's instructions.

15. Incubate the membrane completely with substrate for 1-5 minutes (adjust time for more sensitive ECL substrates e.g. SuperSignal West Femto Chemiluminescent Substrate [Pierce]).

16. Expose the membrane to autoradiography fi lm in a dark room or read using a chemiluminescence imaging system.

17. Line up the developed fi lm in the correct orientation to the blot and mark the bands of the MW ladder directly onto the fi lm. It is also advised to add notes such as lane content, fi lm exposure time and ECL properties.

Tip 7: Use multiple exposure lengths to determine the optimal exposure time. Use fl uorescent markers as a guide for blot-film orientation.

Appendix

Solutions

4X SDS sample buffer (50ml)	
150mM Tris•HCl (pH 7.0) (1M stock)	7.5ml
25% glycerol	12.5ml
12% SDS	6.0g
0.02% Bromophenol Blue	10mg
5% β-mercaptoethanol	3.0ml
Add ddH_2O to 50ml, aliquot and store at -20°C.	

1X TBST (1000ml)	
20mM Tris-base	2.42g
150mM NaCl	8.76g
50mM KCl	3.73g
0.2% Tween-20	2 ml
Adjust pH to 7.6	
Add ddH2O to 1000ml	

Wet transfer buffer (1000 ml)	
25mM Tris-base	3.03g
192mM Glycine	14.4g
20% Methanol 200ml	200ml
Add ddH2O to 1000ml	

Semi-dry transfer buffer (1000ml)	
48mM Tris-base	5.81g
39mM Glycine	2.93g
0.0375% SDS	0.375g
20% Methanol	200ml
Add ddH_2O to 1000 ml	

SDS-PAGE gel recipes

For target proteins with MWs between 20 and 200 kDa, make a conventional SDS-PAGE gel using the following recipes in the table below.

Add: Room 703, Building 6, No. 333 Guiping Rd., Xuhui District, Shanghai City, P.R. China 200233
Tel/ Fax: +86 021-54651191 **Product Inquiry:** market@yhsbio.com
Placing Orders: order@yhsbio.com **Technical Support:** support@yhsbio.com
www.yhsbio.com

Select the percentage of gel you require using the MW of your target protein.

	Separating Gel (mls, total 10 ml)				Stacking Gel (mls)			
MW of target protein (kDa)	80-200	35-100	25-60	20-40	4ml	6ml	8ml	
Gel percentage	8%	10%	12%	15%	4%			Gel percentage
ddH_2O	4.6	4.0	3.3	2.3	2.9	4.3	5.7	ddH_2O
30% Acrylamide	2.7	3.3	4	5	0.4	0.8	1.1	30% Acrylamide
4x Separating/Stacking buffer	2.5	2.5	2.5	2.5	0.5	0.8	1	4x Separating/Stacking buffer
10% APS	0.1	0.1	0.1	0.1	0.04	0.06	0.08	10% APS
TEMED	0.01	0.01	0.01	0.01	0.004	0.006	0.008	TEMED

Buffers

4x Separating buffer recipe (250 ml)

1.5M Tris HCl (pH 8.8), 0.4% SDS Dissolve 46.75g of Tris in 100 ml of ddH_2O, pH slowly to 8.8 with concentrated HCl, add 1g SDS and dissolve thoroughly. Adjust fi nal volume to 250ml with ddH2O.

4x Stacking buffer recipe (250ml)

1.0 M Tris HCl (pH 6.8), 0.1% SDS Dissolve 15.13g of Tris in 100ml ddH_2O, pH slowly to 6.8 with concentrated HCl, add 0.25g SDS and dissolve thoroughly.Adjust final volume to 250ml with ddH_2O.

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