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WESTERN BLOTTING

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

1. SDS PAGE:

a. 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.

b. Prepare samples in microfuge tubes. Add **4X SDS sample buffer** so the total protein amount is 30–50 µg per sample (according to the protein amount measured by Bradford or BCA protein assay).

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

d. Set up electrophoresis apparatus and immerse in **1x running buffer**. Remove gel combs and cleanse wells of any residual stacking gel.

e. 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 first experiment and adjust as necessary after assessing the initial target signal.

2. Protein transfer:

a. 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 filter papers and sponges in **transfer buffer** as well.

b. 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.

Tip 4

If target MW is larger than 100 kDa, 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 buffer to facilitate transfer is preferable.

Tip 5

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

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3. Immunoblotting:

a. 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**.

b. 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.

c. 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 at room temperature, or overnight at 4°C.

d. Wash membrane three times with **1x TBST** for 10 minutes each.

e. 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.

f. 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, 0.02% NaN_3 could be included in the antibody dilution buffer. NB: Not suitable for use with secondary antibody solutions.

4. Signal detection:

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

b. 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]).

c. Expose the membrane to autoradiography film in a dark room or read using a chemiluminescence imaging system.

Tip 7

Use multiple exposure lengths to determine the optimal exposure time.
Use fluorescent markers as a guide for blot-film orientation.

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

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Solutions

4X SDS sample buffer	
150 mM Tris•HCl (pH 7.0) (1M stock)	15 ml (of a 1M stock)
25% glycerol	25 ml
12% SDS	12 g
0.05% Bromophenol Blue	0.05g
6% β-mercaptoethanol	6 ml
Add ddH_2O to 100ml, aliquot and store at -20°C.	

1X TBST	
20 mM Tris-base	2.42 g
150 mM NaCl	8.76 g
50 mM KCl	3.73 g
0.2% Tween-20	2 ml
Adjust pH to 7.6	
Add ddH_2O to 1000ml	

Wet transfer buffer	
25 mM Tris-base	3.03 g
192 mM Glycine	14.4 g
20% Methanol	200 ml
Add ddH_2O to 1000ml	

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

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

Separating gel (mls, total 10 ml)				
MW of target protein (kDa)	80-200	35-100	25-60	20-40
Gel percentage	8%	10%	12%	15%
ddH_2O	2.1	1.5	0.8	0
30% Acrylamide	2.7	3.3	4	5
2x Separating buffer	5.0	5.0	5.0	5.0
10% APS	0.1	0.1	0.1	0.1
TEMED	0.01	0.01	0.01	0.01

Stacking gel (mls)			
	4 ml	6 ml	8 ml
MW of target protein (kDa)	–	–	–
Gel percentage	4%	4%	4%
ddH_2O	1.4	2.1	2.7
30% Acrylamide	0.5	0.8	1.1
2x Stacking buffer	2.0	3.0	4.0
10% APS	0.04	0.06	0.08
TEMED	0.004	0.006	0.008

2x Separating Buffer Recipe (makes 1000ml)	
Tris HCl (pH 8.8)	90.8 g
SDS	2.0 g
Dissolve compounds thoroughly. Adjust pH slowly to pH 8.8 with concentrated HCl, then add ddH_2O to 1000ml.	

2x Stacking Buffer Recipe (makes 1000ml)	
Tris HCl (pH 6.8)	30.35 g
SDS	2.0 g
Dissolve compounds thoroughly. Adjust pH slowly to pH 6.8 with concentrated HCl, then add ddH_2O to 1000ml.	

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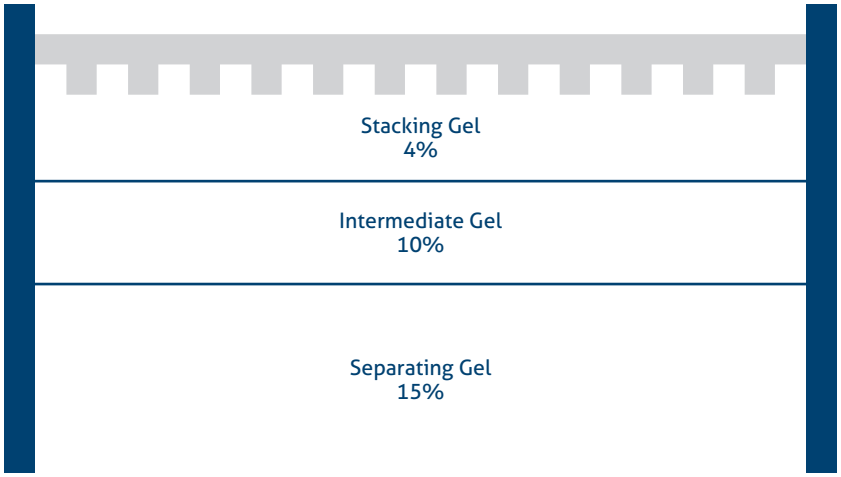
SDS PAGE Gel Recipes

1x Running Buffer Recipe (makes 1000ml)	
Tris-base	1.51 g
Glycine	7.5 g
SDS	0.5 g
Dissolve compounds thoroughly, then add ddH_2O to 1000 ml.	

Tricine Gel Recipe

For target protein with MWs of less than 20 kDa, a tricine gel system will obtain higher resolution and is highly recommended. Make three layers of tricine gels as laid out in the following table and diagram. Apply specific tricine gel running buffer to the running system and perform transfer as usual.

Items	Stacking	Intermediate	Separating
Gel percentage	4%	10%	15%
Gel volume	2 ml	3 ml	6 ml
38% Glycerol	–	–	1.6
ddH_2O	1.4	1.2	–
30% Acrylamide	0.3	0.8	2.7
3.0 M Tris HCl (pH 8.5)	–	1	2.14
1.0 M Tris HCl (pH 6.8)	0.3	–	–
10% SDS	0.02	0.03	0.06
10% APS	0.02	0.03	0.06
TEMED	0.002	0.003	0.003



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