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Protocol for Western Blotting

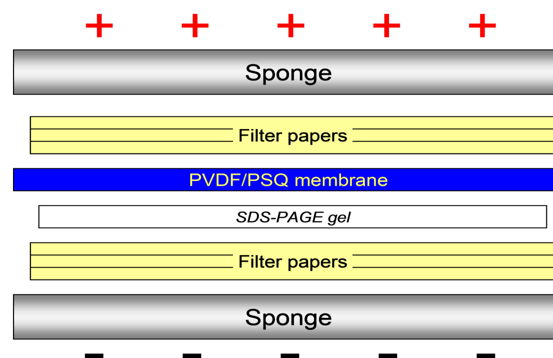
RED TEXT indicates important optimized settings

SDS-PAGE separation

1. Make separation gel (SDS-page gel in Tris-Glycine system) with a gradient from 8-14% .
2. Prepare 30 μg HEK-293 lysate with RIPA buffer by mixing 4X SDS sample buffer with lysate sample according to the protein concentration measured by Bradford or BCA protein assay.
3. Heat at 95-100°C for 5 min. Set up the electrophoresis apparatus with SDS-PAGE gel.
4. Load samples and protein markers onto the gel. Set 80 V to run the stacking gel, increase to 120 V for the separation gel till the end.

Electrotransfer

5. Soak PVDF membrane with pore size 0.45 um in methanol for 30 sec and then in the transfer buffer. Soak the filter papers and sponges in the transfer buffer as well.
6. Sequentially assemble the transfer sandwich according to the illustration and make sure no bubbles are trapped. Apply Wet transfer systems at 180mA for 90 min with Wet transfer buffer.



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Immunoblotting

7. After transferring, wash the membrane twice with distilled water.
8. Block membrane with 1X TBST containing 5% nonfat dry milk with agitation at room temperature for 1 h.
9. Dilute primary antibody (18420-1-AP, P62/SQSTM1 Rabbit PolyAb) 1:1000 in blocking solution. Incubate membrane with the primary antibody with agitation at room temperature for 1.5 hours. Wash membrane 3 times with 1X TBST for 10 min each.
10. Incubate the membrane with the HRP-conjugated secondary antibody diluted at 1:5000 in blocking solution at room temperature for 1 h. Wash membrane 3 times with 1X TBST for 10 min each.

Signal Detection (Chemiluminescence system)

11. Prepare ECL substrate according to the manufacturer's instructions.
12. Incubate the membrane with a substrate for 1-5 min.
13. Exposure autoradiography film in the dark or try a chemiluminescence imaging system. Check a few different exposure times to obtain the optimal image for quantification (30-300 sec).
14. Remember to mark the protein MW markers on the film.

Solutions

RIPA buffer	1000 ml
50 mM Tris·HCl, pH 7.4 (1M stock)	50 ml
150 mM NaCl	8.76 g
1% Triton X-100 or NP-40	10 ml
0.5% Sodium deoxycholate	5 g
0.1 % SDS	1 g
1 mM EDTA (0.5 M stock)	2 ml
Add ddH2O to 1000 ml	

Add PMSF to 1mM and protease inhibitors (1 mM Sodium Orthovanadate) freshly before use.

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4X SDS sample buffer	**50 ml**
250 mM Tris•HCl (pH 7.0) (1M stock)	12.5 ml
40% glycerol	20 ml
5% SDS	2.5 g
0.02% Bromophenol Blue	10 mg
5% β-mercaptoethanol	2.5 ml
Add ddH2O to 50ml, aliquot and store at -20°C	

1X TBST	**1000 ml**
20 mM Tris-base	2.4 g
150 mM NaCl	8.7 g
0.2% Tween-20	2 ml
Adjust pH to 7.6	
Add ddH2O to 1000ml	

1X Running buffer	**1000 ml**
12.5 mM Tris-base	1.51 g
100 mM Glycine	7.5 g
0.05% SDS	0.5 g
Add ddH2O to 1000 ml	

Semi-dry transfer buffer	**1000 ml**
48 mM Tris-base	5.81 g
39 mM Glycine	2.93 g
0.0375% SDS	0.375 g
20% Methanol	200 ml
Add ddH2O to 1000 ml	

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

Tricine gel running buffer	**1000 ml**
100 mM Tris-base	12.1 g
100 mM Tricine	17.9 g
0.1% SDS	1 g
Add ddH2O to 1000ml	

Related Products

Product Name	Catalog No.	Size	Application
HRP-conjugated AffiniPure Goat Anti-Mouse Ig(G+L)	SA00001-1	100 µl	ELISA; WB; IHC/ICC
HRP-conjugated AffiniPure Goat Anti-Rabbit Ig(G+L)	SA00001-2	100 µl	ELISA; WB; IHC/ICC

ELISA:Enzyme-linked immunosorbent assay; WB:Western Blotting; IHC:Immunohistochemistry; ICC:Immunocytochemistry;

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