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China: service@ptglab.com

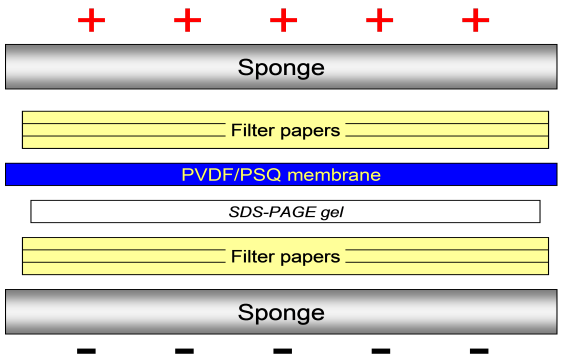
Protocol for Western Blotting

SDS-PAGE separation

1. Make appropriate percentage of separation gel according to the MW of target proteins. Related recommendations and routine recipes of separation/stacking gels are presented in the end.
    - *Tip 1: Tris-Tricine gels separate low MW proteins(less than 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 30 μg cell/tissue 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 gels or Tricine gels.
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.
    - *Tip 3: Load enough sample for the first trial, and adjust experiment system after getting target signal.*

Electrotransfer

5. In general, PVDF membranes (or PSQ membrane with 0.22 μm micropore when MW target is less than 30 kDa) are recommended. Soak membranes in methanol for 30 sec and then into 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 semi-dry or wet transfer systems according to manufacturer's instructions.
    - *Tip 4: If target MW is larger than 100 kDa, wet transfer at 4 °C overnight is suggested instead of semi-dry method. Additional 0.1% SDS in the wet transfer buffer is recommended to facilitate transferring.*



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Immunoblotting

7. After transferring, wash the membrane twice with distilled water. If desired, stain the membrane with commercial Ponceau red solution for 1 min to visualize the protein bands, mark the MW markers on the membrane with a pencil then wash up the staining with 1X TBST.
8. Block with 1X TBST containing 5% nonfat dry milk (or 1-5% BSA for phospho-epitope antibodies) with agitation at room temperature for 1 h.
9. Dilute primary antibody with appropriate dilution (1:500 - 1:10,000) in blocking solution. Optimal dilution should be determined in prests. Incubate PVDF 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.
    - *Tip 5: Do not let the membrane dry for the entire process. 0.2% NaN3 could be included in the primary antibody dilution for preservation but never in the secondary antibody dilution.*

Signal Detection

11. Prepare ECL substrate according to the manufacturer's instructions.
12. Incubate the membrane completely with substrate for 1-5 min.
13. Expose autoradiography film in a dark room or exposure membrane under a chemiluminescence imaging system. Perform multiple exposures to determine the optimal one. (60-180 Sec exposure to get the fig)
14. Remember to mark the protein MW markers on the film.

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Solutions

4X SDS sample buffer	50 ml	1X TBST	1000 ml
250 mM Tris•HCl (pH 7.0) (1M stock)	12.5 ml	20 mM Tris-base	2.4 g
40% glycerol	20 ml	150 mM NaCl	8.7 g
5% SDS	2.5 g	0.2% Tween-20	2 ml
0.02% Bromophenol Blue	10 mg	Adjust pH to 7.6	
5% β-mercaptoethanol	2.5 ml	Add ddH2O to 1000ml	
Add ddH2O to 50ml, aliquot and store at -20°C			

1X Running buffer	1000 ml	Semi-dry transfer buffer	1000 ml
12.5 mM Tris-base	1.51 g	48 mM Tris-base	5.81 g
100 mM Glycine	7.5 g	39 mM Glycine	2.93 g
0.05% SDS	0.5 g	0.0375% SDS	0.375 g
Add ddH2O to 1000 ml		20% Methanol	200 ml
		Add ddH2O to 1000 ml	

Wet transfer buffer	1000 ml	Tricine gel running buffer	1000 ml
25 mM Tris-base	3.03 g	100 mM Tris-base	12.1 g
192 mM Glycine	14.4 g	100 mM Tricine	17.9 g
20% Methanol	200 ml	0.1% SDS	1 g
Add ddH2O to 1000 ml		Add ddH2O to 1000ml	

Related Products in PROTEINTECH

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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Recipes for SDS-PAGE Gel

A. For MW of target protein between 20-200 kDa, conventional SDS-PAGE gel could be configured following recipes in the table below.

ITEMS	Separation Gel (10ml)				Stacking Gel (ml)		
MW of target protein	80-200 kDa	35-100 kDa	25-60 kDa	20-40 kDa	4ml	6ml	8ml
Gel percentage	8%	10%	12%	14%	4%	4%	4%
ddH_2O	4.6	4.0	3.3	2.3	2.9	4.3	5.7
30% Acrylamide	2.7	3.3	4	5	0.5	0.8	1.1
1.5 M Tris•HCl (pH8.8)	2.5	2.5	2.5	2.5	-	-	-
1.0 M Tris·HCl (pH6.8)	-	-	-	-	0.5	0.8	1
10% SDS	0.1	0.1	0.1	0.1	0.04	0.06	0.08
10% APS	0.1	0.1	0.1	0.1	0.04	0.06	0.08
TEMED	0.01	0.01	0.01	0.01	0.004	0.006	0.008

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B. For MW of target protein less than 20 kDa, tricine gel system is greatly suggested to obtain higher resolution. Make three layers of tricine gels as in the following table, apply specific tricine gel running buffer to the running system and perform transfer as usual.

ITEMs	Separation	Intermediate	Stacking
Gel percentage	15%	10%	4%
Gel volume	6 ml	3 ml	2ml
38% Glycerol solution	1.6	-	—
ddH_2O	—	1.2	1.4
30% Acrylamide	2.7	0.8	0.3
3.0 M Tris·HCl (pH8.5)	2.14	1	-
1.0 M Tris•HCl (pH6.8)	—	—	0.3
10% SDS	0.06	0.03	0.02
10% APS	0.06	0.03	0.02
TEMED	0.003	0.003	0.002