Western Blotting(WB) Protocol

-Instruction

Western blot is a technique used to identify and locate proteins based on their ability to bind to specific antibodies. Firstly, proteins are separated by SDS-PAGE gel depending on Charge effects and Molecular sieve effects. Secondly, separated proteins are stained to the surface of PVDF or nitrocellulose membrane. Finally, the interesting antigen which absorbs on the membrane interacts with specific antibody, and can be detected by detection regents. Western blotting can give you information about the size of your protein (with comparison to a size marker or ladder in kDa), and also give you information on protein expression (with comparison to a control such as untreated sample or another cell type or tissue).

-Solution and reagents

30% Acrylamide (makes 500ml)	
Acrylamide	**145g**
Bis-acrylamide	**5g**
Fully dissolve the compounds at 37℃. Then add ddH2O to 500ml and preserve it in a brown bottle at RT.	
pH6.8 Tris-Hcl (makes 500ml)	
Tris-base	**90.75g**
Fully dissolve the compounds. Adjust pH to 6.8 by concentrate HCl. Then add water to make up to 500ml.	
pH8.8 Tris-Hcl (makes 500ml)	
Tris-base	**90.75g**
Fully dissolve the compounds. Adjust pH to 8.8 by concentrate HCl. Then add water to make up to 500ml.	
Sample 6×loading buffer (makes 15ml)	
Tris-Hcl (pH 6.8)	**1ml**
SDS	**6g**
Bromophenol blue	**0.09g**
Glycerol	**3ml**

DTT	**1.35ml**

Fully dissolve the compounds. Then add water to make up to 15ml

Concentrate gel (makes 4ml)	
30% Acrylamide	**0.67ml**
Tris-HCl (pH 6.8)	**0.5ml**
ddH_2O	**2.7ml**
10%SDS	**40μl**
10%APS	**40μl**
TEMED	**6μl**

0.1g SDS is dissolved in 1ml ddH_2O by heating to 56℃. 0.1g APS is dispersed in 1ml ddH_2O and should be prepared when it will be used. 10% APS and TEMED are an accelerator which should be rapidly mixed and poured into the device.

Separate gel (makes 10ml)				
MW of target protein	**70-200**	**25-70**	**20-35**	**10-20**
Gel percentage	**8%**	**10%**	**12%**	**15%**
30% Acrylamide	**2.5ml**	**3.33ml**	**4ml**	**5ml**
Tris-HCl (pH 6.8)	**2.5ml**	**2.5ml**	**2.5ml**	**2.5ml**
ddH_2O	**4.85ml**	**4.02ml**	**3.3ml**	**2.3ml**
10%SDS	**100μl**	**100μl**	**100μl**	**100μl**
10%APS	**100μl**	**100μl**	**100μl**	**100μl**
TEMED	**6μl**	**6μl**	**6μl**	**6μl**

0.1g SDS is dissolved in 1ml ddH_2O by heating to 56℃. 0.1g APS is dispersed in 1ml ddH_2O and should be prepared when it will be used. 10% APS and TEMED is an accelerator which should be rapidly mixed and poured into the device.

Runing buffer (makes 1000ml)	
SDS	**1g**
Tris-base	**3g**
Glycine	**14.4g**

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Fully dissolve the compounds. Then add water to make up to 1000ml.

For the protein weight lower than 20kd, Trincine buffer is recommend, the recipe as follow:

Runing buffer for small molecular proteins			
Cathode solution (makes 1000ml)		**Anode solution (makes 1000ml)**	
Tricine	**17.9g**	**Tris-base**	**24.228g**
Tris-base	**12.1g**		
SDS	**1g**		
Fully dissolve the compounds. Then add water to make up to 1000ml.			

Transfer buffer (semi-dry, makes 1000ml)	
Tris-base	**2.43g**
Glycine	**11.26g**
Methanol	**200ml**
Fully dissolve the compounds and mix up. Then add water to make up to 1000ml. Preserved in dark.	

Transfer buffer (wet , makes 1000ml)	
Tris-base	**3.03g**
Glycine	**14.4g**
Methanol	**200ml**
Fully dissolve the compounds and mix up. Then add water to make up to 1000ml. Preserved in dark.	

IF the weight of target protein is bigger than 100kD, 0.04% SDS is recommended to be added in transfer buffer and it is recommended to reduce the percentage of methanol to 10% or lower.

Blocking buffer (makes 1000ml)	
Casein	**10g**
Fully dissolve the compounds and mixed up. Then add 1×PBS to make up to 1000ml.	

Antibody dilution buffer (makes 1000ml)	
Blocking buffer	**1000ml**
Tween-20	**500ul**
Fully dissolve the compounds and mix up.	

-Sample preparation

一. Preparation for lysate of cell culture

1. Remove media and wash cells with sterile PBS.

2. Collect cells into PBS and pellet in a centrifuge.

3. Lyse cells by adding RIPA buffer (1ml for 1×10^7 cells) which is mixed with fresh 1/100 volume protease inhibitors in advance. Keep on ice.

4. Sonicate the cell suspension to shear DNA and complete cell lysis (the time of sonication is varied from the type of cell), then incubate on ice for 15-20min.

5. Spin at 12,000rmp for 15-20min at 4℃.

6. Siphon the supernatant into a new tube. Keep on ice all the time.

7. Measure the protein content in the supernatant by Bradford assay or BCA assay.

8. Add appropriate 6×sample loading buffer to the supernatant according to the protein content and the amount of protein needed to load on.

9. Boil samples for 5min at 100℃ (unless noted otherwise). Cool on ice.

10. Centrifuge for 5min, reserved in -20℃.

二. Preparation for lysate of tissue

1. Dissect the needed tissues on ice and wash away the excess blood.

2. Place the fresh tissue in a clean mortar, and grind tissue by liquid nitrogen. Collect the tissue powder into a precooling tube.

3. Add appropriate RIPA buffer according to the amount of tissue (1ml for 0.1mg tissue).

4. Sonicate the supernatant on ice for proper time, which varies from different tissue, then incubate on ice for 15-20min.

5. Spin at 12,000rmp for 15-20min at 4℃.

6. Siphon the supernatant into a new tube. Keep on ice all the time.

7. Measure the protein content in the supernatant by Bradford assay or BCA assay.

8. Add appropriate 6×sample loading buffer to the supernatant according to the protein content and the amount of protein needed to load on.

9. Boil samples for 5min at 100℃ (unless noted otherwise). Cool on ice.

10. Centrifuge for 5min, reserved in -20℃.

NOTE: 1. Keep the whole process on ice. Avoid the degradation of protein.

2. Make sure that the interesting protein expresses in the sample which you choose.

-Preparation of gel

1. Assemble the glass plates and spacers (1.5 mm thick).

2. Pour an agarose plug (1-2 mm).

3. Pour the running gel to about 1 cm below the wells of the comb (~20 ml). Avoid the bubble.

4. Seal with 1 ml water-saturated 1-butanol.

5. When gel has set, pour off the butanol and rinse with deionized water.

6. Pour the stacking gel (~5 ml) and insert the comb immediately.

7. When the stacking gel has set, place in gel rig and immerse in buffer.

8. Prior to running the gel, flush the wells out thoroughly with running buffer.

9. You need to choose a perfect gel content depending on the size of your target protein. Follow the table below.

Protein size（kD）	Gel percentage
10-20	15%
20-55	12%
25-70	10%
70-200	8%

-Running the gel

1. After flash spinning the samples to remove impurities, 10-30μg of total protein is recommended to load into the wells. The recommended quantity of purified protein is 10-100ng. Loading quantities should be adjusted according to the result.

2. Molecular weight markers should be included in a lane to indicate the protein of interest.

3. Run with constant voltage (voltage set at 120V or lower). To obtain a better experimental result, 80V is recommended when the whole protein migrates in concentration gel. While the proteins migrate into separation gel, voltage should be boosted up.

4. Usual running time is about 1.2h. However, the protein which weighs lower then 20kD should shorten the running time according to the interesting protein. While the protein is bigger then 100kD, running time should be extended to get a better separation of protein.

-Transfer and Blocking

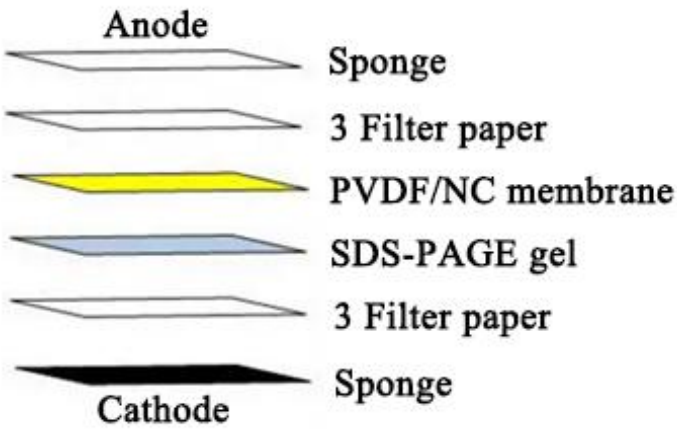
1. Dilute the primary antibody in blocking buffer and incubate 1-3h at room temperature up to overnight at 4℃. Low temperature and long term are recommended. Overnight incubation yields improved antibody binding.

2. Wash the membrane in PBS for 10min for three times and apply the diluted conjugated secondary antibody in blocking buffer (as per manufacturer's instructions) and incubate 1h at room temperature.

3. Wash the blot in PBST three times for 30min.

4. Be careful to separate the separation gel.

5. Make the "sandwich" by filter paper-gel- filter paper, keep the "sandwich" hydrated and avoid bubble between it. Prepare the "sandwich" as follow：



6. Assemble the transfer device according to manufacturer's manual. Transfer proteins to nitrocellulose or PVDF membrane. 0.22μm membrane is recommended for the protein which weighs lower than 20kD.

7. There are usually two devices that you can choose in the light of actual conditions, wet transfer or semi-dry-transfer. Both wet transfer and semi-dry-transfer can work well either for high molecular weight proteins or low molecular weight proteins. But semi-dry-transfer can yield higher background staining.

8. Rinse the blot in PBS for approximately 5 minutes.

9. Block the membrane using 1% casein in PBS (may add 1% BSA or 5% non-fat dry milk) for 1h at room temperature.

-Antibody incubation

1. Dilute the primary antibody in blocking buffer and incubate 1-3h at room temperature up to overnight at 4℃. Low temperature and long term are recommended. Overnight incubation yields improved antibody binding.

2. Wash the membrane in PBS for 10min for three times and apply the diluted conjugated secondary

antibody in blocking buffer (as per manufacturer's instructions) and incubate 1h at room temperature.

3. Wash the blot in PBST three times for 30min.

-F Detection

1. Apply the detection reagent of choice in accordance with the manufacturer's instructions. Chemiluminescent reagents should be mixed directly prior to use to minimize signal reduction due to light exposure and degradation over time. The exposure time requires a little increase by attempt to get a better film.