

Western Blot Protocol

Follow current protocols on SDS-PAGE gel running and Western Transfer.
(Refer to Table 1 for the effective range of separation of SDS-PAGE)

Step-by-step procedure:

1. Add adequate amount of 5% non-fat milk/PBST (0.2%) blocking buffer and leave the sample at room temperature for 1 hour or under 4°C overnight. (Keep the membranes at -20°C no longer than a week if the membranes will not be used immediately.)
2. Dilute the primary antibody with fresh blocking buffer to the designated concentration. Remove the membrane from the previous blocking buffer and add the diluted primary antibodies to the membrane. Incubate at 4°C overnight. (Refer to Table 2 for dilution factors).
3. Wash sample with PBST (0.2%) for 10 minutes. Repeat 3 times.
4. Add adequate amount of Goat Anti-Mouse IgG (H&L)-HRP Conjugate secondary antibody (Catalog No. PAB0096) (refer to Table 2 for dilution factors). Leave the sample at room temperature for 1 hour.
5. Wash sample with PBST (0.2%) for 10 minutes. Repeat 4 times.
6. After washing the sample, place PVDF membrane into a sealable bag and add freshly prepared chemiluminescent reagents into the bag to coat the entire membrane.

For maximum sensitivity, PIERCE SuperSignal[®] West Femto Maximum sensitivity substrate is recommended. The reagent should be in 1:1 dilution of reagent A and B as working solution (following strictly to the provider's instructions). Add working solution to the membrane and seal the bag. Spread the chemiluminescent reagent around so it can be distributed evenly onto the membrane. (Chemiluminescent reagent must be spread out evenly otherwise parts of the membrane will be over-exposed when the photograph is taken.) 0.6 mL of working solution is used for membrane size 8 cm x 10 cm.

7. Take photographs immediately with CCD camera at 5-second, 20-second, 1.5-minute, and 5-minute intervals, in order to acquire proper exposure image.

Strong signals may intensify into blackout signals with hollow circles. In this case, dilute the secondary antibody.

Table 1. Effective Range of Separation of SDS-PAGE

Acrylamide Concentration (%)	Linear Range of Separation (kDa)
15	10-43
12	12-60
10	20-80
8	36-94
6	57-212

Table 2. Recommended Antigen and Antibody amount use in Western Blot ^a

Antigen		Primary Antibody Dilutions				Secondary Antibody Dilutions ^b
Type of Antigen	Amount (µg/lane)	Poly-sera	Hybridoma Supernatant	Ascites	Purified Ascites	
Cell/Tissue Lysate	25 ~ 50	1:500 ~ 1:1000	Undiluted ~ 1:5	1:500 ~ 1:1000	1 ~ 5 (µg/ml)	1:2500 ~ 1:5000
Purified Protein	0.1 ~ 0.2	1:1000 ~ 1:2500	Undiluted ~ 1:5	1:500 ~ 1:1000	1 ~ 5 (µg/ml)	1:5000 ~ 1:10000

Note:

^a The information provide in this table shall be used only as a guide, each investigator should determine the optimal antigen amount and antibody dilution for their own specific research application.

^b Goat Anti-Mouse IgG (H&L)-HRP Conjugate secondary antibody (Catalog No. PAB0096).

Equipment(s)

- Shaker (TKB OS701)
- AutoChemi System (UVP)

Reagents

- **Blocking buffer/dilution buffer:**

Weigh non-fat milk 5 g and dissolve in 100 mL 1X PBST (0.2%) to a final mixture of 5% non-fat milk/PBST (0.2%).

- **(10X) PBST (phosphate buffer saline):**

NaCl	(0.13 M x 10, Merck 6404)	75.9 g
NaH ₂ PO ₄ ·H ₂ O	(0.01 M x 10, Merck A429146 335)	13.8 g

Add 800 mL ddH₂O. After the salts have dissolved, use NaOH liquid to adjust the solution to pH 7.0 and make the final dilute solution to 1,000 mL. The solution becomes 10X PBS. Dilute

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the solution with ddH₂O to the final 1X PBS prior application.



- **PBST (0.2%) (Phosphate buffer saline and 0.2% Tween 20):**

1X PBS	1 L
Tween 20	2mL

Dilute 10X PBS with ddH₂O to a final 1X concentration. Add Tween 20 [0.2% (v/v)] to final PBST (0.2%).

- **Anti-Mouse IgG Secondary antibody:**

Goat Anti-Mouse IgG (H&L)-HRP Conjugate secondary antibody (Abnova, Cat. No. PAB0096).

- **Chemiluminescent reagent:**

SuperSignal[®] West Femto Maximum Sensitivity Substrate (PIERCE, Cat. No. 34094, 34095 & 34096)

Reagent must be freshly prepared each time before application.

References:

1. AutoChemi system instruction manual
2. ECL substrate kit.