Western Blot Protocol

(Tissue Lysate)

SECTION 1 – Equipment & Reagent Preparation

1.1 Equipment(s)

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

1.2 Blocking Buffer (also 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%).

1.3 Sample Buffer (Bio-Rad, Catalog #: 161-0747)

1.4 RIPA Lysis Buffer (Thermo Fisher, Catalog #: 89900)

1.5 (10X) PBST (phosphate buffer saline)

NaCl	(0.13 M x 10, Merck 6404)	75.9 g
$NaH_2PO_4 \cdot H_2O$	(0.01 M x 10, Merck A429146 335)	13.8 g

Add 800 mL ddH_2O. 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 the solution with ddH2O to the final 1X PBS prior application.

1.6 PBST (0.2%) (Phosphate buffer saline and 0.2% Tween 20)

1X PBS	1 L
Tween 20	2 mL

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

1.7 Anti-IgG Secondary antibody

Use one of the following secondary antibodies according to the host species of which primary antibody raised against (please refer to **Table 2** for detail information):

a. Goat Anti-Mouse IgG (H&L)-HRP Conjugate secondary antibody (Abnova, Catalog #: PAB0096)

b. Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson Immunoresearch Laboratories, INC., Catalog #: 115-035-062).

c. Goat Anti-Rabbit IgG (H+L), Peroxidase Conjugated (Pierce, Catalog #: 31460)

1.8 Chemiluminescent reagent

SuperSignal® West Femto Maximum Sensitivity Substrate (PIERCE, Catalog #: 34094, 34095 & 34096)

Reagent must be freshly prepared freshly each time before application.

SECTION 2 - Loading Sample Preparation

Tissue lysate:

1. Re-suspend cell pellet in the tube, count cell number.
2. Add 3 mL modified ice-cold RIPA Lysis Buffer into 10^8 cells (Approximately comes from 1g of tissue or 1 mL of cell culture). Incubate on ice for 40 min, vortex 4 to 6 times during incubation.
3. Centrifuge at 4°C, 27,000 g for 30 min. Aspirate supernatant into a new tube.
4. Add 4x sample buffer to supernatant at 1:3 volume ratio. Then incubate in water bath at 95°C for 10 min..
5. Dilute the sample to proper sample amount/concentration for loading. Refer to Table 2.

SECTION 3 - Assay Protocol

Please follow **Current Protocols** on SDS-PAGE gel running and Western Transfer. (Refer to **Table 1** for the effective range of separation of SDS-PAGE)

2.1 Add adequate amount of blocking buffer and incubate the membrane at room temperature for 1 hour or under 4°C overnight. Please keep the membranes at -20°C no longer than a week if it will not be used immediately.

2.2 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. Please refer to Table 2 for dilution factors.

2.3 Wash membrane with PBST (0.2%) for 10 minutes. Repeat 3 times.

2.4 Add adequate amount of anti-IgG secondary antibody (please refer to Table 2 for detail).

Leave the membrane at room temperature for 1 hour.

2.5 Wash membrane with PBST (0.2%) for 10 minutes. Repeat 4 times.

2.6 After washing, place 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 (Strictly follow 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.

2.7 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 band. If happen, user may 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 amount and Antibody dilution use in Western Blot [a].

Antigen	Type of Antigen	Tissue Lysate
	Loading amount	25 ~ 50 µg/lane
Abnova's Primary Antibody (recommended dilution or concentration) [e]	Mouse polyclonal antibody	1:500 ~ 1:1000
	Mouse MaxPab®	1:500 ~ 1:1000 (or 1 µg/ml for purified Mouse MaxPab®)
	Rabbit MaxPab®	1:1000 (or 1 µg/ml for purified Rabbit MaxPab®)
	Hybridoma cell culture supernatant (Mouse Ig)	Undiluted ~ 1:5
	Ascites (Mouse Ig)	1:500 ~ 1:1000
	Monoclonal antibody (Mouse Ig)	1 ~ 5 µg/ml
Secondary Antibody (dilutions or concentration)	Goat Anti-Mouse IgG (Abnova) [b]	1:2500 ~ 1:5000
	Goat Anti-Mouse IgG (Jackson Immunoresearch) [c]	1:5000 ~ 1:10000
	Goat Anti-Rabbit IgG (Pierce) [d]	1:7500

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 (Abnova Corp., Catalog No. PAB0096).

[c] Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson Immunoresearch Laboratories, INC., Catalog No. 115-035-062).

[d] When the primary antibody is raised in rabbit, please use Goat Anti-Rabbit IgG (H+L), Peroxidase Conjugated (Pierce, Cat. No. 31460)

[e] Please follow the instruction on the product sheet, if it has the recommend dilutions of the primary antibody.