





## Protocol

1. Remove blot from the transfer apparatus and soak in transfer buffer. Under the hood, pour the Millennium Enhancer solution into a new container. For a 50 cm<sup>2</sup> blotting membrane, use 10 ml Millennium Enhancer, and the solution can be re-used 4 times without losing its enhancing effect. Pick the membrane from the transfer buffer and drain the remaining buffer on the membrane. For best results, do not let the membrane dry completely. Soak the membrane in the Millennium Enhancer solution, agitate for 2 min, then remove the membrane and submerge it in 1 x TTBS solution.
2. Block the membrane with 5% non-fat dry milk in 1 x TTBS solution for 30 min at room temperature with agitation.
3. Prepare antibody binding working buffer by diluting the supplied 20x antibody binding stocking buffer 20 times with ddH<sub>2</sub>O. Make an appropriate dilution of primary antibody in antibody binding working buffer; 10 ml of primary antibody solution volume is suggested for a 50-cm<sup>2</sup> membrane. Remove the blocking reagent and add the primary antibody solution. Incubate the blot with agitation for one hour at room temperature to overnight at 2-8°C.
4. Wash the membrane in 1 x TTBS for 5 min, repeat 3-4 times.
5. Incubate blot with the appropriate HRP-conjugate secondary antibody solution, diluted with antibody binding working buffer (e.g., 1:5,000 for anti-Mouse IgG) for 1 hour at RT with agitation. Ten ml secondary antibody solution is suggested for membrane size of 50 cm<sup>2</sup>.
6. Repeat step 4 to remove unbound HRP-conjugate.
7. Prepare substrate-working solution by mixing equal volume of Substrate Buffer A and Buffer B right before developing, 2 ml substrate working solution (mixture of 1 ml Buffer A and 1 ml Buffer B) is suggested for a 50 cm<sup>2</sup> membrane.
8. Incubate blot with substrate working solution for 1-5 min.
9. Place membrane between plastic protection sheets or transparent plastic wrap, and mount inside a film cassette with the protein side facing up.
10. Place a piece of film on top of the membrane, let it expose for an appropriate time, and develop the film. A recommended initial exposure time is 1 min.
11. The blot can be re-developed if necessary.
12. After stripping, the blot can be re-probed.

### Re-developing Method

Soak membrane in 1 x TTBS solution at 2-8°C O/N. On the second day, (incubate with secondary antibody and apply wash buffer, this step is optional) add substrate and expose on film.

### Stripping Method

Use stripping buffer M if the mild condition is sufficient; and use stripping buffer H if more stringent stripping conditions are necessary.

1. Soak the membrane in stripping buffer and incubate at 50°C for 30 min with occasional agitation (incubate for a longer time or raise temperature to 70°C if the membrane is not completely stripped).
2. Wash the membrane twice in a large volume of 1 x TTBS for 10 min at room temperature.
3. Repeat the immunoblotting procedure from the blocking step.

### Trouble Shooting

#### 1. General Problems

##### 1.1 No signal or weak signal

- Proteins did not transfer properly to membrane

**Cytomol Unimed** 592-10 Weddle Dr., Sunnyvale, CA 94089 USA  
Tel: 408-992-0400 Fax: 877-713-8298 E-mail: [info@cytomol.com](mailto:info@cytomol.com) Website: [www.cytomol.com](http://www.cytomol.com)

- Not enough protein loaded on the gel
- Target protein degradation occurred due to improper storage of blot
- The concentration of primary or secondary antibody used was too low
- The blocking buffer used was not correct, and antigen was covered
- Substrate had lost activity

**1.2 High background**

- The concentration of the primary or secondary antibody used was too high
- Too much protein loaded on the gel
- Insufficient blocking
- Insufficient washing
- The level of Tween-20 in blocking buffer was too low
- Membrane problems: e.g., PVDF membrane was not wetted thoroughly or dried in processing
- Transfer buffer been contaminated

**1.3 Reverse image on film**

- Too much antigen
- Too much HRP in the system

**2. Kit-specific Problems**

**2.1 Little or no Enhancing Effect**

- The antigen level was too high, the signal was already saturated
- Too much buffer remained on the membrane before Millennium Enhancer treatment or Millennium Enhancer has been re-used too many times, Millennium Enhancer has been diluted too much and lost its activity
- The membrane was not treated long enough in Millennium Enhancer solution
- Specific nature of antigen

**2.2 High Background**

- A general problem, see above
- The signal was magnified too highly, use the kit without Millennium Enhancer treatment

**Related Products**

Western Blot, Protein array, Total protein, Compartment Proteins

**Appendix**

**Preparation of solutions not supplied with kit:**

Solution	Preparation	Stability/temperature	Notes
<b>1 x TTBS</b>	Add 6.05 g Tris base (50 mM), 8.76 g sodium chloride (150 mM) to 800 ml distilled water, adjust pH to 7.5 with HCl; adjusted to 1 liter with distilled water. Add Tween-20 to 0.1% (v/v)	3 months at RT	Do not use sodium azide as an antimicrobial agent as it inhibit HRP
<b>Blocking Solution</b>	Weigh 5 g of non-fat dry milk and dissolve it in 100 ml 1 x TTBS solution	Freshly made suggested	Can be Stored at 2-8°C O/N
<b>Stripping Buffer M</b>	100 mM Glycine, pH 2.7	1 month at RT	Mild stripping buffer
<b>Stripping Buffer H</b>	62.5 mM Tris-HCl, pH 6.7 with 2% SDS and 100 mM 2-Mercaptoethanol	1 month at RT	Harsh stripping buffer