PROTOCOL FOR WESTERN BLOTTING (Wet Transfer)

(Quan Kang 7/10/2003; updated by Yang Bi 05/03/2008, commented by TCH)

1) Run the samples in SDS-PAGE as usual.
2) Prepare and mark the membrane (right size, PVDF or Immobilon-P membrane) with a pencil; then soak it in Methanol for a few minutes, and rinse it with distilled water.
3) Equilibrate the membrane, pads, filter papers (4 pieces) and transfer foam in Transfer Buffer (store in **4°C**) for 5 minutes.
4) Assemble transfer sandwich in the following order: **black frame** (negative electrode) >> foam >> **3** pieces of filter papers >> SDS-PAGE gel >> membrane >> **3** pieces of filter papers >> foam >> **red frame** (positive electrode). Make the marked side of membrane face the gel. Put it into transfer tank.
5) Transfer the gel at 80V for 90mins in cold room. Alternatively, use ice bag and magnet stirring bar to transfer the gel in room temperature.
6) Disassemble the gel pack, and use a pencil to mark the well imprints on the membrane. Block the membrane with **Blocking Solution** (SuperBlock Blocking Buffer from PIERCE) on a rocking platform at 4°C overnight or at room temperature for 1-2 hrs. (**Note**: Overnight blocking yields a lower background).
7) Prepare the primary antibody solution with **Blocking Solution** (usually 1:200-1:1000, depending antibody reactivity). Usually 5-10ml solution is enough for a small container.
8) Place the membrane into the primary antibody solution and rock the container for 1hr at room temperature (or at 4°C overnight).
9) Wash the membrane with **TSBT** for 5 minutes x 3 (rocking).
10) Prepare the secondary antibody (i.e., **HRP-conjugated** secondary antibody, or **biotin-conjugated** secondary antibody) solution with TSBT (usually 1:2000 – 1:5000) and incubate the membrane at room temperature for 30 minutes on a rocking platform.
11) Wash the membrane with TSBT 5 minutes x 3.
12) (**Optional**: If the secondary antibody is labeled with biotin, incubate the membrane with the streptavidin-HPR conjugate at 1:2000 to 1:5000 for 20-30 min at room temperature, followed by washing it with TBST 5min x 3).
13) Prepare the ECL working solution of the SuperSignal **West Pico** Chemiluminescent Substrate (PIERCE, Cat# 34080) by mixing **Reagent 1** and **Reagent 2** at 1:1 ratio (vol : vol). 2ml per membrane are sufficient.
14) Incubate the membrane in the ECL working solution for 60 seconds.
15) Place the membrane face-down on the Kodak ImageStation, follow the instructions to visualize the protein band (**Note**: Remember to check binning for X and Y, with the blank filter slot).

TBST (Tris-Buffered Saline-Tween-20):

Final concentration:
10mM Tris-HCl (pH8.0)

150mM NaCl
0.05% Tween-20 (after all and water are mixed, add tween-20)

Transfer Buffer:

For 4L Buffer
800ml Methanol
12.12g Tris
57.63g Glycine
Add ddH2O to 4 liters
Kept at 4^0C

COMMENTS:

Our **default protocol** is to use PIERCE's SuperSignal **West Pico** Chemiluminescent Substrate (PIERCE, Cat# 34080). However, if the protein is in low abundance and/or the antibody has weak reactivity, you can redevelop the membrane using the **West Femto** substrate kit. According to PIERCE, the Femto substrate is 1,000 times more sensitive than the Pico substrate. The downsides of Femto substrate are two-fold: potentially higher background and more expensive.

Here is a statement from PIERCE: "Chemiluminescence yields the greatest sensitivity of any available detection method. Using HRP as the enzyme label and SuperSignal West Femto Chemiluminescent Substrate (**PIERCE Cat# 34095**), lower detection limits in the low femtogram range are possible because the enhancers in this substrate greatly intensify the emitted light and extend the signal duration. SuperSignal West Femto Maximum Sensitivity Substrate provides the ultimate sensitivity for Western blotting. This substrate provides the highest possible sensitivity, allowing users to see protein bands that were never visualized before".

PIERCE website links:
http://www.piercenet.com/Products/Browse.cfm?fldID=01041101 and http://www.piercenet.com/Products/Browse.cfm?fldID=01041106