Institut für HIV Forschung
SOP#11-01 **(Juli 2016 - BS)**

WESTERN BLOT

Reagent	Manufacturer	Order Number
Glycine	Roth	3790.2
Methanol	Roth	X948.1
Milk Powder	Roth	T145.1
10X SDS Running Buffer	Bio-Rad	1610732
Tris-HCl	Sigma	T3253-250G
Tris-base	Sigma	T1503-1KG
Nitrocellulose	Carl Roth	0031.1
Gel Blotting Paper	Carl Roth	AE19.1
Primary Antibody		
Secondary Antibody		
DAB Substrate Kit	Thermo	34002
PBS	Life Technologies	14190-144
Tween 20	Carl Roth	9127.1

Transfer, Washing, and Blocking Buffers:

Transfer Buffer [semi-dry] (1L):

Reagent	Stock Concentration	Volume of Stock	Final Concentration
Tris base pH 8.0	250mM	100mL	25mM
Glycine	1.9M	100mL	190mM
Methanol	100%	200mL	20%
dH2O	-	Up to 1000mL	-

**We can a 10X stock of Tris-Glycine on the shelf. Use 100mL of this instead of the separate, above listed Tris base/Glycine solutions.*

Or when creating it using powder

Reagent	Stock Concentration	Amount of Stock/Powder	Final Concentration
Tris base pH 8.0	-	5.81g	48mM
Glycine	-	2.93g	39mM
Methanol	100%	200mL	20%
SDS	10%	4mL	0.04%
dH2O	-	Up to 1000mL	-

Washing Buffer – PBS-T (500mL):

Reagent	Stock Concentration	Volume of Stock	Final Concentration
PBS	-	500mL	-
Tween-20	100%	500ul	0.1% (v/v)

Blocking Buffer – PBS-T+5% (w/v) Milk Powder (500mL):

Reagent	Stock Concentration	Volume of Stock	Final Concentration
TBS	-	500mL	-
Tween-20	100%	500ul	0.1%
Milch Powder	100%	25g	5% (w/v)

Protocol:

0. First you must cut nitrocellulose and 6x blotting paper in the same size as your gel. The Mini-Protean Gels from Bio-Rad require a cut-out of **6cm x 9cm**
    a. These can vary in size by a little, but try not to have more than a 5-10mm error.
    b. You need one piece of nitrocellulose and 6x blotting paper for each gel you are transferring
1. Soak the blotting paper and gel in Transfer Buffer for 5min
2. Soak the nitrocellulose in dH2O for 5min
    a. This activates the nitrocellulose and prepares it to accept proteins. When using PVDF membrane, instead soak it in MeOH
3. Pre-wet the electrodes (giant metal sheets inside semi-dry blotter) by covering the inside of it with paper towels and spraying dH2O on them until thoroughly wet. Close the blotter so both electrodes get damp
4. Remove the paper towel from the electrodes and stack 3 pieces of pre-soaked blotting paper on top of one another on the bottom electrode. Make sure they are as aligned as possible
5. Remove air from between the pieces of **blotting paper** by taking a 50mL conical and rolling it over them 4-5 times
6. Place the **membrane** on top of the pre-soaked stack of blotting paper
7. Carefully place your **gel** on top
8. Finally top the sandwich off with the other **3 pieces of blotting paper**. Again, remove excess air by rolling a 50mL conical over it.
9. Close the blotter and connect the cables (match those colors!) to the grey power box. For the Mini-Protean Gels, set it to a constant amperage of 45mA for 1hr
    a. Most transfers are complete in an hour but larger proteins or proteins from native gels may require additional time
    b. The maximum current should not be more than 0.8mA/cm^2. The amperage is decided by finding the area of your gel (in cm^2) and multiplying by 0.8mA.
10. After the run, turn off the power supply and disconnect the cables

11. Open the blotter and carefully remove the top half of the sandwich. Verify that your proteins transferred by looking to see if the protein ladder is now on the membrane.
12. Remove the membrane and set it inside the white plastic tray located near the gel station or inside a clean pipette tip lid. Let the membrane dry for 1hr-overnight
13. Once dry, block the membrane 1hr at room temperature by pouring ~50mL Blocking Buffer into the container the membrane is in. Place the container on a shaker and turn it on a low setting
    a. Note that if you plan on staining with two different primary antibodies than it is suggested that the membrane is cut prior to blocking as once the membrane is wet it becomes difficult to cut. Be sure to mark each section now to avoid confusion later
14. Prepare 5mL of the primary antibodies dilution(s) during the blocking step by diluting the primary antibody in Blocking Buffer in a 50mL conical. Primary antibodies should be titrated but the datasheet typically has a suggested dilution.
15. Once the blocking is finished, use the tweezers (Pinzette) to move each section of the membrane to the conical containing its respective antibody solution.
16. Make sure the lids are on tight and rotate the conicals on the rotator in the cold room overnight
17. The next morning, remove the membranes from their conicals and put them in a clean container
    a. Note that these diluted antibodies can be kept at 4C for ~2 weeks and used 1-2 more times
18. Wash the sections by shaking them for 5-10min with ~50mL Washing Buffer 3 times
    a. While holding your membrane sections, pour the Washing Buffer in the sink and add fresh buffer
19. Prepare 20mL of the secondary antibodies dilution(s) during the last washing step by diluting the secondary antibody in Blocking Buffer in a 50mL conical. Secondary antibodies should be titrated but the datasheet typically has a suggested dilution.
20. After disposing of the last wash buffer, add 50ml of Blocking Buffer and shake for 5min
21. Pour off the Blocking Buffer and move the membrane sections to separate containers
22. Pour the secondary antibodies solutions into their respective containers with their sections. Shake containers at room temperature for 1hr
23. Repeat step 18
24. Develop your signal(s)
    a. Currently we have only the DAB substrate kit which reacts with HRP conjugated secondary antibodies to form a brown stain that you can visualize. To use this:
        i. Mix 2.5mL DAB substrate with 22.5mL of Peroxide Buffer Solution in a 50mL conical
        ii. Collect your sections into one container and pour the DAB solution onto your sections
        iii. Develop 5-15min
    b. Another (more sensitive) method involves using enhanced chemiluminescence (ECL) or image scanner for colorimetric detection