

## NeoClone Western Blot Protocol

Objective: This protocol describes NeoClone's Procedure for performing Western Blots

### Materials needed:

Unstained gel from Gel Electrophoresis Procedure ("Gel")  
Nitrocellulose transfer membranes ("Nitrocellulose") [Schleicher & Schuell: Protran 0.45 $\mu$ m BA85]  
Filter Paper  
Target Antibody in Ascites  
Alkaline Phosphatase conjugated Anti-mouse IgG ("Secondary Antibody") [Chemicon AP308A]  
Paper towels ("Towels")

### Solutions:

Deionized H<sub>2</sub>O ("dH<sub>2</sub>O")  
NuPAGE® transfer buffer (20X) ("Transfer Buffer") [Invitrogen NP0006] stored at 4°C  
1% dry skim milk in PBS ("BLOTTO") store at 4°C  
5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium pre-mix substrate ("BCIP/NBT") [Sigma B-3804]  
Tris-buffered saline with Tween ("TBST") [see Appendix]  
Alkaline phosphatase buffer (AP Buffer) [See Appendix]

### Equipment needed:

Xcell SureLock Mini-Cell system ("Mini-Cell System") [Invitrogen EI0001, EI0020, EI0002]  
BioRad Power Pac 300 ("Power Pack") [BioRad Laboratories]  
Mini-incubation tray ("Tray") [Fisher NC9412314]  
Refrigerator [Frigidare/American]  
Lab-Line shaker ("Shaker") (Fisher, Model # 1314)

### Blot Set-up:

1. Put an orientation mark on the Nitrocellulose to match the Gel unless a prestained marker was used.
2. Wet the nitrocellulose with Transfer Buffer.
3. In a Tray, containing Transfer Buffer, set up the Mini-Cell System: Support - Sponge - Filter paper - Gel - Nitrocellulose - Filter Paper - Sponge - Support. Make sure that the orientation marks correspond on the Gel and the Nitrocellulose. Avoid air bubbles between the Gel and the Nitrocellulose.
4. Place the Blot setup in the Mini-Cell System containing Transfer Buffer, with the **Nitrocellulose toward the anode (red)**.

### Transfer Blot:

1. Using the Mini-Cell System & the Power Pack, transfer the Blot at 30V for 2 hours.
2. **Turn off the Power Pack** & remove the Blot from the Mini-Cell System and, in the Tray, block with BLOTTO. Blocking is usually complete after 1-2 hr at room temperature, but it is often convenient to simply leave the blot in BLOTTO overnight at 4° C (Refrigerator). (The blotted Nitrocellulose can be dried and used at a later time.)

3. Dilute the Target Antibody 1:1000 in BLOTTO (check product data sheet for recommended dilution). React with blotted protein for about 1hr at room temperature with gentle shaking on the Shaker at speed 3 (all Shaker steps should be at this speed).
4. Wash the Blot 3 times with TBST in the Tray. Washes are about 5min each on the Shaker at speed 3 (all the Shaker steps should be at this speed).

Secondary Antibody:

1. Dilute the Secondary Antibody 1:2000 with BLOTTO.
2. React the diluted Secondary Antibody with BLOTTO in the Tray for about 1hr at room temperature shaking on the Shaker at speed 3 (all the Shaker steps should be at this speed).
3. Decant the 2° antibody/BLOTTO mix in the sink. Wash the Blot 5 times with TBST. (Add TBST to the Blot, shake, and decant. Washes are about 5min each with gentle shaking on the Shaker at speed 3 (all the Shaker steps should be at this speed).
4. Wash the Blot 1 time in AP Buffer for about 5min with gentle shaking.
5. React with BCIP/NBT substrate with gentle shaking on the Shaker at speed 3 (all the Shaker steps should be at this speed) until bands appear. **This time will vary.**
6. Before the background gets too dark, wash the Blot with 2 changes of dH<sub>2</sub>O. Put the Blot on Towels to dry.

## Appendix

### **Buffers and Reagents**

#### Blocking Agent

1% protein solutions are generally made up in phosphate-buffered saline or Tris-buffered saline. (BLOTTO is 1% nonfat dry milk in PBS).

#### Tris-buffered Saline with Tween 20 (TBST)

10 mM Tris HCl, pH7.4	10ml 1M Tris-HCl, pH7.4
150 mM NaCl	37.5ml 4M NaCl
0.1% mM Tween 20	1ml Tween 20
q.s. to 1 liter with dH <sub>2</sub> O	

#### Alkaline Phosphatase Buffer (AP Buffer)

100 mM Tris HCl, pH9.5	100 ml 1M Tris-HCl, pH9.5
100 mM NaCl	25 ml 4M NaCl
5 mM MgCl <sub>2</sub>	5 ml 1M MgCl <sub>2</sub>
q.s. to 1 liter with dH <sub>2</sub> O	