

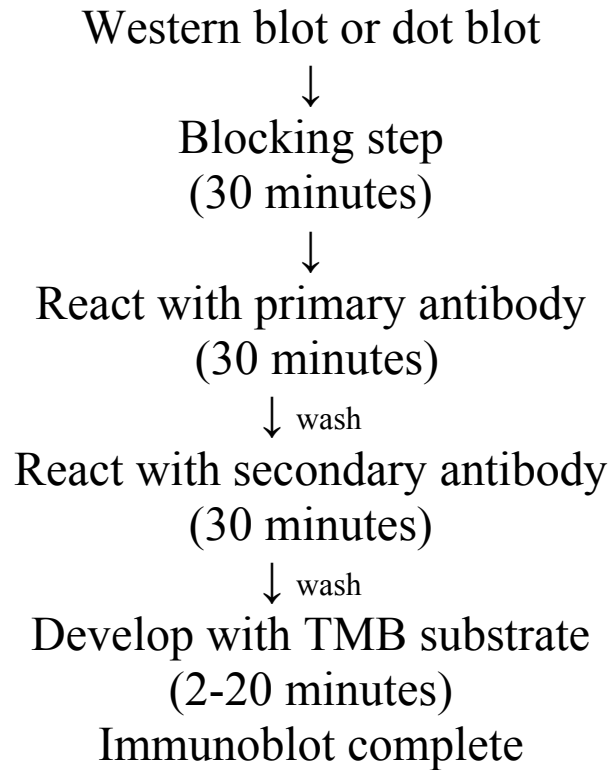
## Introduction

Western blotting is a fast and sensitive method for the identification and characterizing proteins. Western blotting combines the resolution of SDS-PAGE with the specificity of immunodetection using antibodies. Generally western blotting involves electro blotting proteins that have been separated on a SDS-PAGE gel onto a membrane (nitrocellulose or PVDF). After Transfer it is important to block all unreacted sites The membrane is first probed with a primary antibody to detect a specific protein. Then the blot is probed with a secondary antibody that will recognize the primary antibody. The secondary antibody is conjugated with some type of reporter molecule (Usually either ALPH or HRPO) .The membrane can be visualized after the addition of a chromogenic substrate. The substrate used is 3,3',5,5'-tetramethylbenzidine (TMB). When HRPO reacts with hydrogen peroxide in the TMB substrate, an insoluble blue dye is precipitated onto the site where the enzyme labeled antibody is bound to the membrane.

The use of western blotting is a convient alternative to techniques such as ELISA. Unlike ELISA western blotting allows the researcher to identify the molecular weight of the protein of interest.

Separation via electrophoresis involves denaturation of the protein. Therefore it is critical that the primary antibody used for the analysis can detect an epitope that is denaturation resistant.

## Flow Diagram of the EZ-Blot Kit Procedure



## Preparation of Reagents

1. For all concentrate solutions: Allow Concentrates to warm to room temperature. If crystals appear in any of the concentrates, mix to redissolve. If necessary warm to 37° C and mix to redissolve. Dilute 30X concentrate solution 1/30 with reagent quality water. (i.e. mix 1 ml of the coating solution concentrate to 29 mls of reagent quality water). Dilute 10 X concentrate solution 1/10 with reagent quality water. (I.e. mix 1 ml of the coating solution concentrate to 9 mls of reagent quality water). Only dilute what will be used within 2 weeks. After 2 weeks discard.
2. HRP labeled secondary antibody: We recommend a dilution range of 1:500 to 1:2500 in Western Blocker/Antibody Diluent solution.
3. Positive Control: Add 5-10 ul of the positive control to one of the lanes when performing electrophoresis.

## Instructions

The following method is recommended as a guideline for the EZ-Blot western blotting kit.

1. After transferring the protein to the membrane immerse the membrane in the blocking solution using 0.18 ml/cm<sup>2</sup>. Incubate for 30 minutes with gentle agitation.
2. Transfer the membrane to a solution of the primary antibody diluted in the 1 X blocking solution using 0.18 ml/cm<sup>2</sup>. Incubate for 30 minutes with gentle agitation. The

- appropriate dilution should be determined by the user and will vary on the antibody. A good starting point would be 1 ug/ml for the primary antibody. Too much antibody will result in non-specific bands.
3. Wash the membrane three times with the 1X wash solution using 0.22ml/cm<sup>2</sup> for 5 minutes with gentle agitation.
  4. Transfer the membrane to a solution of the secondary antibody diluted 1 to 500 In 1 X blocking/diluent solution using 0.18 ml/cm<sup>2</sup>. Incubate the membrane for 30 minutes with gentle agitation.
  5. Wash the membrane three times with the 1X wash solution using 0.22ml/cm<sup>2</sup> for 5 minutes each with gentle agitation.
  6. Add 5 mls of the TMB substrate using 0.05 ml/cm<sup>2</sup>. Incubate until color develops ( usually 2 to 20 minutes)
  7. Wash the membrane with 3 changes of deionized water using 0.22ml/cm<sup>2</sup> 5 minutes each with gentle agitation to stop development.

## **Additional Notes**

- These instructions are to be used as a guideline. The activity of your own antibodies may require you to deviate from these instructions
- Nylon membranes are not recommended to be used with this kit unless other blocking and washing buffers are used since our buffers contain Tween -20
- If you are seeing too much background on your blots try diluting your primary antibody until right before your background starts to drop off to remove the background. We use Casein and Tween 20 as blocking agents in our buffers. If background is still an issue you may want to use a different

- blocking agent such as BSA Serum or another commercially available agent.
- Some primary antibodies do not bind well in the presence of mild detergents like Tween -20. In this case you may want to replace the diluents buffer with a TBS or PBS with BSA or a 10% normal goat serum
  - Make sure to have enough liquid to cover the membrane. Never allow the membrane
  - Do not touch the membrane with your skin. Always use gloves.
  - Use a rocking platform set at a low speed for gentle agitation in all the incubation steps
  - All incubations occur at room temperature.
  - Avoid using solution containing sodium azide or other inhibitors of HRPO

## Troubleshooting Guide

### **Problem: Little or no signal**

*Solution A: Incomplete transfer of protein.* Make sure you followed the protocols included with the transfer equipment.

*Solution B: Poor binding of the primary antibody.* Increase the concentration of the primary antibody. Or increase the incubation time of the primary antibody to several hours or overnight. Increase the incubation temperature to 37 C°.

*Solution C: Poor binding of the HRPO labeled secondary antibody.* Make sure the primary antibody is the appropriate species for this kit. Increase the concentration of the secondary

antibody. Make sure to include 5 ul of the control IgG as a sample in your western blot or dot blot to ensure that the components of the kit are performing.

*Solution D: Inactive HRPO.* Make sure all buffers are free of sodium azide which is a strong inhibitor of HRPO activity.

**Problem: Too much signal**

*Solution A:* Too much protein on the membrane. Check the protein concentration of the sample. For best results use 10 ug of total protein per well.

*Solution B:* Primary antibody is too high of a concentration. Dilute the primary antibody.

*Solution C:* Too long in substrate solution. Stop the color reaction as soon as the desired endpoint is reached. Generally in a couple of minutes but no longer than 20 minutes.

**Problem: High background**

*Solution A:* Insufficient blocking. Make sure the blocking step was not omitted.

*Solution B:* Insufficient washing. Increase the number of wash steps and the volume of wash buffer for each step.

## References

*Antibodies, A Laboratory Manual.* Ed Harlow and David Lane, eds. Cold Spring Harbor Press. 1988.

*Current Protocols in Molecular Biology.* J Ausebel, et al, eds. John Wiley and Sons, New York.

*Antibodies, A Practical Approach.* 2<sup>nd</sup> Edition. Catty, D., ed. IRL Press, Oxford, England. 1990. Volumes I and II represent a detailed and complete reference for most current antibody techniques.

