

Optimize antigen and antibody concentrations for Western blots

TR0024.1

Introduction

Because every Western blot involves a combination of antibody and antigen interactions, no one antibody concentration exists and optimization is essential. Appropriate primary and secondary antibody concentrations depend on each antibody's specific activity and specificity for its antigen, as well as the amount of antigen present in the sample. The specific activity of secondary antibodies varies among manufacturers. Re-optimization is necessary when one or more of the experimental variables such as the antigen, primary or secondary antibody, or substrate is changed.

When using Thermo Scientific SuperSignal® Chemiluminescent Substrates, concentrations of the antigen, primary and secondary antibodies that are too high or too low can cause a variety of undesirable results. The following list includes possible results from a Western blot that would require optimization of antigen/antibody concentrations:

- Weak signal
- Signal fades quickly
- No signal with no background
- High background, white bands (reversed image)
- Orange or brown spots on the membrane
- Nonspecific bands
- Diffuse bands
- Blotchy or speckled background

The optimal antigen and antibody concentrations can be determined by performing a Western blot with varying concentrations. An easier and quicker method is to perform a dot blot procedure using this protocol.

Dot Blot Procedure for Optimization of Antigen and Antibody Concentrations

A. Materials Required

- Nitrocellulose membrane cut into 1 cm x 8 cm strips that may be treated differently to determine source background
- Antigen-containing sample, diluted in phosphate buffered saline (PBS), Tris buffered saline (TBS) or similar buffer
- Primary and HRP-conjugated secondary antibodies
- Blocking Buffer: test the blocking buffer that resulted in high background in the original procedure
- Wash Buffer, either PBS or TBS, with Tween®-20 Detergent added to a final concentration of 0.05%
- Substrate working solution (WS): chemiluminescent Substrate for HRP (e.g., SuperSignal Substrate)
- Film or CCD camera apparatus to detect chemiluminescence

B. Procedure

1. Prepare dilutions of the protein sample in either TBS or PBS. The proper dilution will depend on the antigen concentration present in the sample. Because the concentration of the antigen of interest is often not known, test a wide range of dilutions. SuperSignal West Substrates have low-picogram to low-femtogram detection sensitivity, depending on the substrate used. Sample dilutions can range from the low-microgram to low-femtogram levels.
2. Prepare nitrocellulose membranes strips, labeled with pencil for each primary/secondary antibody concentration condition to be tested. The number of strips needed depends on how many different dilutions of primary and/or secondary antibody will be screened. Typically one or two dilutions of the primary antibody are tested with two or three

different dilutions of the secondary antibody. For example, with SuperSignal West Pico Substrate, the following dilution ranges are appropriate to test:

- 1/1,000 primary with 1/50,000 secondary
- 1/1,000 primary with 1/100,000 secondary
- 1/5,000 primary with 1/50,000 secondary
- 1/5,000 primary with 1/100,000 secondary

3. Place dry nitrocellulose membrane strips on a paper towel. Dot antigen dilutions onto the strips, using the smallest possible volume for each dot (1-5 μ l) to keep the dot sizes as small as possible. To dot >5 μ l samples, dot 3-5 μ l aliquots on the same spot, allowing each aliquot to dry for 2-5 minutes before dotting the next one. Allow the membranes to dry 10-15 minutes or until no visible moisture remains.
4. Block the nonspecific sites on the nitrocellulose membranes by incubating in Blocking Buffer for 1 hour at room temperature with shaking.
5. Prepare dilutions of primary antibody in Wash Buffer containing 1/10 volume of Blocking Buffer and apply to the membrane strips. Incubate for 1 hour at room temperature with shaking. Those strips that are treated with the same primary antibody dilution may be incubated together in the same tray or tube.

Suggested dilutions (from a 1 mg/ml antibody stock solution) for SuperSignal West Substrates:

- SuperSignal West Pico Substrate: Primary Antibody 1:1,000-1:5,000
- SuperSignal West Dura Substrate: Primary Antibody 1:5,000-1:50,000
- SuperSignal West Femto Substrate: Primary Antibody 1:5,000-1:100,000

6. Wash membrane strips 4 $\times$ 5 minutes in Wash Buffer using as large a volume of wash buffer as possible.
7. Prepare dilutions of the secondary antibody in Wash Buffer containing 1/10 volume of Blocking Buffer. Add the diluted secondary antibody to the membrane strips and incubate for 1 hour with shaking. Those strips that are treated with the same secondary antibody dilution may be incubated together in the same tray or tube.

Suggested dilutions (from a 1 mg/ml antibody stock solution) for SuperSignal West Substrates:

- SuperSignal West Pico Substrate: Secondary Antibody 1:20,000-1:100,000
- SuperSignal West Dura Substrate: Secondary Antibody 1:50,000-1:250,000
- SuperSignal West Femto Substrate: Secondary Antibody 1:100,000-1:500,000

8. Wash the membrane again as described in Step 6.
9. Prepare the Substrate working solution (WS) by mixing equal volumes of the SuperSignal Substrate Luminol/Enhancer Solution and the Stable Peroxide Solution. Prepare a sufficient volume to ensure that all strips are completely wetted with substrate and the blot does not dry out during incubation. Recommended volume: 0.125 ml/cm² of blot surface.
10. Incubate the membrane strips in the Substrate WS for 5 minutes.
11. Remove membrane strips from substrate and place in a plastic sheet protector or other protective wrap.
12. With the protein side up, place wrapped strips against film and expose 30-60 seconds. Exposure time can be varied to obtain optimum results; if the first exposure is not sufficient, try 2-5 minutes. Alternatively, a CCD camera or other imaging device can be used; however, these devices may require longer exposure times.

On an optimized blot, the light generated by the reaction of the Substrate WS should last for 6-24 hours, depending on the particular product used. The blot can be re-exposed to film or an imaging device as needed to obtain the optimal results. Longer exposure times may be necessary as the blot ages. If optimal results are not achieved, repeat the above procedure using different antigen and/or antibody dilutions.

Related Thermo Scientific Products

- Nitrocellulose membrane (e.g., Product No. 88013)
- BupH™ Phosphate-buffered saline packs (PBS, Product No. 28374)
- BupH Tris-buffered saline packs (TBS, Product No. 28376)
- Primary and HRP-conjugated secondary antibodies (see our web site for a complete listing)
- SuperBlock® Blocking Buffer (Product No. 37515 or 37535)
- StartingBlock™ Blocking Buffer (Product No. 37538 or 37542)
- Blocker BSA (Product No. 37520 or 37525)
- Peroxidase Suppressor (Product No. 35000)
- Surfact-Amps® 20 (Product No. 28320), 10% solution of Tween®-20 Detergent
- Pierce ECL Western Blotting Substrate (Product No. 32106, 32209, 32109)
- SuperSignal West Chemiluminescent Substrates for HRP (Product No. 34080, 34075, 34095)
- CL-XPosure™ Film (Product No. 34090 or 34091)

*SuperSignal® Technology is protected by U.S. Patent #6,432,662.

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Current versions of product instructions are available at www.thermo.com/pierce. For a faxed copy, call 800-874-3723 or contact your local distributor.

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