TECH TIP # 22

Thermo SCIENTIFIC

Determine source of nonspecific background signal in Western blots detected using SuperSignal® Chemiluminescent Substrates

TR0022.1

Introduction

Western blotting procedures depend on appropriate interactions among multiple components, including membrane, antigen, blocking buffer, primary and secondary antibodies, and substrate. In a particular experiment, any one of these interactions can contribute to excessive background signal in Western blotting experiments. This Tech Tip describes several different dotblot procedures that can be used to determine the source of background caused by nonspecific interactions among system components in a Western blotting procedure that uses a chemiluminescent substrate for horseradish peroxidase, such as Thermo Scientific SuperSignal or Pierce® ECL Substrates.

Before concluding that nonspecific background is caused by an incompatibility between Western blot components and using the procedure described here, make sure that the correct primary and secondary antibody dilutions have been used in the original procedure. SuperSignal Chemiluminescent Substrates are very sensitive, and using inappropriate antibody dilutions can result in a variety of unexpected results:

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

Consider optimizing primary and HRP-conjugated secondary antibody dilutions (see Tech Tip # 24) before or in addition to testing for nonspecific background signal.

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 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

Procedure

Cut a piece of membrane into four strips that may be dotted, treated and detected using the following 4 conditions:

A. Strip 1

Incubate clean, untreated membrane strip in Substrate WS for 5 minutes. Place the wet membrane in plastic and expose it to film. If signal is detected, there is a problem either with the membrane or the substrate. Perform the Darkroom Test (described in the instructions for SuperSignal Substrate Products) to determine if the substrate is working properly to detect active HRP enzyme.

B. Strip 2

Block untreated strip of membrane using the Blocking Buffer used in the original Western blotting procedure. Wash the strip (6 × 5 minutes) in Washing Buffer and then incubate it in Substrate WS for 5 minutes. Place the wet membrane in plastic and expose it to film. If signal is detected, the Blocking Buffer contains endogenous peroxidase activity. Correct the problem by using a different blocking buffer (see Related Products section). Alternatively, use Peroxidase Suppressor (Product No. 35000) following the blocking step.

C. Strip 3

Dot 2-5 µl of the primary antibody onto the membrane strip; make one dot using antibody that has been diluted to the appropriate concentration or make several dots of various dilutions in the suggested range for the substrate being used (see instructions for the substrate). Block and wash the membrane (6 × 5 minutes) in Wash Buffer. Incubate the strip in Substrate WS for 5 minutes; then place the wet membrane in plastic and expose it to film. If signal is detected, the primary antibody is contaminated with endogenous peroxide activity. Correct the problem by either changing the primary antibody used or by blocking endogenous peroxidase activity with Peroxidase Suppressor (Product No. 35000) following the primary antibody incubation.

D. Strip 4

Block the untreated strip and wash 6 × 5 minutes in Wash Buffer. Incubate the strip using the secondary antibody dilution and conditions used in the original Western blot procedure that resulted in high background. Wash the strip 6 × 5 minutes in Wash Buffer; then incubate the strip in Substrate WS for 5 minutes, place in plastic and expose to film. If signal is detected, secondary antibody is cross-reacting (nonspecifically binding) to the blocking agent. Correct the problem by using a different blocking buffer. Repeat these steps to test the new blocking buffer.

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.