INSTRUCTIONS

North2South™ Chemiluminescent Substrate for HRP

17295

0911.2

Number	Description
17295	**North2South Chemiluminescent Substrate for HRP**, sufficient substrate for 1000cm^2 of membrane
	Kit Contents:
	North2South Luminol/Enhancer Solution, 50mL
	North2South Stable Peroxide Solution, 50mL
	Storage: Stable for one year at 4°C or six months at room temperature. Products are shipped at ambient temperature.

Introduction

The Thermo Scientific North2South Chemiluminescent Substrate is for detecting nucleic acids in Northern and Southern blot applications. This robust system uses an enhanced luminol substrate for horseradish peroxidase (HRP) that greatly reduces processing and film exposure time. Post-hybridization processing has been reduced from the standard 2.5 hours to 1 hour. Film exposure times range from 30 seconds to 10 minutes with the substrate emitting light with relatively constant intensity for up to 6 hours, allowing for multiple exposures.

Additional Materials Required

- Biotinylated nucleic acid probe (e.g., North2South Biotin Random Prime DNA Labeling Kit, Product No. 17075)
- Positively charged nylon transfer membrane (e.g., Biodyne™ B Membrane, Product No. 77016)
- Streptavidin-HRP Conjugate (Product No. 21124, 21126 or 21127)
- Hybridization Buffer (North2South Hybridization Buffer, Product No. 37549)
- Hybridization Stringency Wash Buffer (e.g., North2South Hybridization Stringency Wash Buffer, Product No. 37555)
- Blocking Buffer (North2South Blocking Buffer, Product No. 37550)
- Wash Buffer (e.g., North2South Wash Buffer, Product No. 37560)
- X-ray film (Product No. 34090 or 34091)
- Equilibration Buffer: 0.28M Tris•HCl, pH 8.0

Procedure for Nucleic Acid Hybridization and Detection

A. Pre-Hybridization and Hybridization

Note: Heat incubator to the appropriate temperature (e.g., 55°C for DNA hybrids or 65°C for RNA:RNA hybrids). Quantitate the probe using a spectrophotometer.[1,2]

1. Equilibrate Hybridization Buffer to room temperature (RT).
2. Place the blot in a container, such as a 50mL centrifuge tube, and add sufficient Hybridization Buffer to completely cover the blot. Use at least 0.1mL per cm^2 of membrane.
3. Seal the container and pre-hybridize the membrane with shaking or rotating for at least 30 minutes. For DNA hybrids incubate at 55°C; for RNA:RNA hybrids incubate at 65°C.
4. While pre-hybridizing, denature the biotinylated DNA probe. Heat the DNA probe at 100°C for 10 minutes and place on ice for 5 minutes.

5. After pre-hybridization, add the denatured biotinylated probe. For RNA probes, add 3-5ng of probe per milliliter of hybridization solution; for DNA probes, add ~30ng of probe per milliliter of hybridization solution.
6. Incubate overnight with shaking or rotating at 55°C for DNA hybrids or 65°C for RNA:RNA hybrids.

B. Stringency Washes

1. On the following day, equilibrate the Hybridization Stringency Wash Buffer to RT.

   Note: Various wash conditions may be used to increase or decrease stringency if required. In general, stringency increases with increasing temperature and decreasing ionic strength.
2. Wash the membrane three times for 15-20 minutes per wash with agitation. Use 0.2mL of Hybridization Stringency Wash Buffer per cm^2 of membrane and perform washes at 55°C for DNA hybrids or 65°C for RNA:RNA hybrids.

C. Probe Detection

Note: Use clean forceps to handle only the corners of the membrane. For optimal results, rinse forceps with ethanol and allow them to dry in-between steps.

1. Decant the Hybridization Stringency Wash Buffer and add sufficient Blocking Buffer to generously cover the membrane. Use at least 0.25mL/cm^2 of membrane. Incubate with shaking or rotating for 15 minutes at RT.
2. Determine the amount of Streptavidin-HRP to add to obtain a **final conjugate concentration of ~30ng/mL (1:30,000 dilution of a 1mg/mL stock)** in the tube with the blot, but do not add it yet. Decant a portion of the Blocking Buffer from the tube containing the membrane into a separate tube.
3. Add the Streptavidin-HRP conjugate from Step 2 to the separated buffer. Add the buffer/Streptavidin-HRP mix to the tube containing the membrane and incubate for 15 minutes at RT with agitation.

   Note: Performing Steps 2 and 3 as described will prevent the undiluted conjugate from coming in direct contact with the membrane, which would produce undesirable results.
4. Wash the membrane with wash buffer four times for 5 minutes each at RT with gentle agitation. Place membrane into a clean wash container for the next step.
5. Add Substrate Equilibration Buffer to the container with the membrane. Use 0.25mL/cm^2 of membrane. Incubate for 5 minutes at RT with agitation.

D. Substrate Development

1. Prepare the Substrate Working Solution by mixing equal volumes of North2South Luminol/Enhancer Solution and North2South Stable Peroxide Solution. Prepare enough solution to completely cover the membrane (i.e., ~0.1mL/cm^2).

   Note: The Working Solution is stable for 6 hours at room temperature. Exposure to the sun or any other intense light can harm the Working Solution. For best results, keep the Working Solution in an amber bottle and avoid prolonged exposure to intense light. Typical laboratory lighting will not harm the Working Solution.
2. Place the moist membrane on a tray or a piece of plastic wrap and cover with the Substrate Working Solution. Incubate for 5 minutes at RT. Make sure the membrane is fully covered with substrate.
3. Drain the substrate from the membrane surface and transfer the moist membrane to a sheet protector, or wrap it in clear plastic wrap. Remove any trapped air bubbles or wrinkles within the plastic wrap and blot any substrate that may have leaked from the edges.
4. Expose the blot to film for 1 minute. Shorter or longer exposures may be required to obtain desired signal.
5. Develop the film according to the manufacturer's instructions.

Note: For images that have been overexposed or show high background and/or speckling, use the Thermo Scientific Pierce Background Eliminator Kit (Product No. 21065).

Note: For best results, use a new membrane for each hybridization procedure. If stripping and reuse of the membrane is required, test the stripping protocol by incubating the stripped membrane in Substrate Working Solution, placing it in a sheet protector and exposing it to film for at least 30 minutes. If no bands are visible, the membrane can be rinsed with Wash Buffer and re-probed.

Troubleshooting

Problem	Possible Cause	Solution
High background	Probe concentration too high	Reduce probe concentration during hybridization
	Too much HRP conjugate used	Use a final conjugate concentration of ~30ng/mL (1:30,000 dilution of a 1mg/mL stock)
	Undiluted probe contacted the membrane	Thoroughly mix the probe in Hybridization Buffer before allowing it to contact the membrane
	Wrong blocking or hybridization buffer was used	Use North2South Buffers (see Additional Materials Required Section)
	Wrong type of membrane was used	Use Biodyne B Nylon Membrane (Product No. 77016)
Speckling background	Precipitate in the HRP conjugate	Filter the conjugate through a 0.2µm filter before use, or centrifuge 1 minute at maximum speed or use Pierce® Background Eliminator
Weak or no signal	Insufficient film exposure	Expose to film for 30 minutes or longer
	HRP conjugate concentration too low or too high	Use a final conjugate concentration of ~30ng/mL (1:30,000 dilution of a 1mg/mL stock)
	Low target concentration	Increase probe concentration during hybridization and/or increase film exposure time
	DNA probe not completely denatured	Heat probe to 100°C (boil) for 10 minutes and chill on ice 5 minutes
Weak or no signal	Poor membrane transfer	Check transfer (blotting) protocol
	Inactive HRP conjugate	Test HRP by spotting 1µL of the HRP conjugate onto a small piece of membrane, wash and detect with the Substrate Working Solution
	Probe is degraded or not labeled	Test probe by spotting it onto membrane and then detect with Streptavidin-HRP as outlined in procedure
	Probe did not hybridize to target	Use North2South Buffers (see Additional Materials Required Section)
		Reduce the hybridization temperature
Clear (ghost) bands	Too much HRP	Dilute the HRP conjugate an additional 25- to 50-fold
Nonspecific bands	Degradation of target nucleic acid	Prevent nuclease contamination by using nuclease-free reagents or nuclease inhibitors
	Too much target nucleic acid present	Decrease the target concentration
	Too much probe was used	Reduce probe concentration during hybridization
	Stringency washes not sufficient	Increase wash stringency by decreasing ionic strength in the wash solution and/or by increasing the temperature

Related Thermo Scientific Products

17075 **North2South Biotin Random Prime DNA Labeling Kit**

77016 **Biodyne Membrane,** 8 × 12cm, 25/package

34090 **CL-XPosure™ Film,** 5 × 7in, 100 sheets/package

21065 **Pierce Background Eliminator Kit,** for reducing background from exposed X-ray film

89880 **Chemiluminescent Nucleic Acid Detection Module**

17097 **North2South Hybridization and Detection Kit**

Cited References

1. Sambrook, J., *et al.* (1989). *Molecular Cloning: A Laboratory Manual,* 2nd ed. Cold Spring Harbor Laboratory Press.
2. Ausubel, F.M., *et al.* (1995). *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc.

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