



DNA MODIFYING ENZYMES

Phototope[®]-Star Detection Kit

Instruction Manual

NEB #N7020S
Store at 4°C

 NEW ENGLAND
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Kit Components:

Streptavidin

1.0 mg/ml (1000X) concentration 1.0 ml

Biotinylated Alkaline Phosphatase

0.4 mg/ml (1000X) concentration 1.0 ml

CDP-Star[®] Reagent

25 mM solution 1.0 ml

CDP-Star[®] Dilution Buffer:

2-amino-2-methyl-1-propanol (25X) concentrate 20 ml

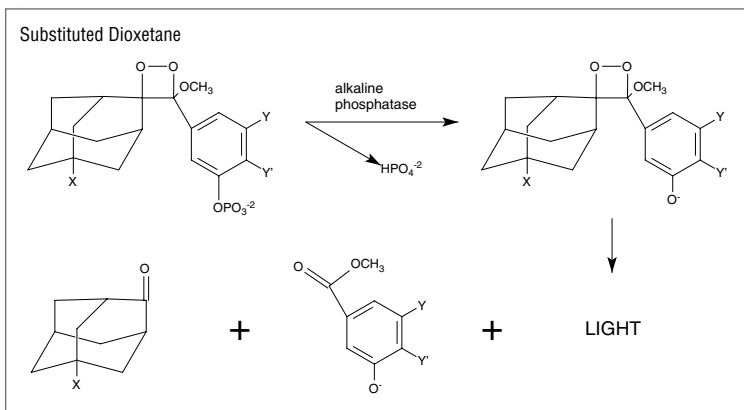
Introduction:

Phototope detection with CDP-Star® is based on chemiluminescence: an enzyme-catalyzed reaction which emits a blue light. This detection method is replacing radioactive detection due to sensitivity, convenience, safety and cost.

In the Phototope®-Star Detection Kit, biotinylated target, on a membrane support, is detected by first exposing the membrane to streptavidin, which binds to the biotinylated target and then biotinylated alkaline phosphatase which binds to the streptavidin. In the final step, the CDP-Star Reagent is added. Alkaline phosphatase catalyzes the removal of the phosphate from the CDP-Star Reagent (phenylphosphate substituted 1,2 dioxetane) to yield a moderately stable intermediate which spontaneously decays, emitting light at 461 nm (1,2). The emitted light is detected by exposing the membrane to x-ray film for 15 seconds to 15 minutes or phospho-imager according to manufacturer's recommendations.

The biotin handle can be either directly on your target DNA or on a biotinylated probe hybridized to your target DNA/RNA. DNA can be labeled by random primer extension reaction (3,4,5) (NEBlot® Phototope Kit, NEB #N7550S), PCR or photo-biotin. Alternatively, custom synthesized oligonucleotide probes can be biotinylated.

Figure 1.



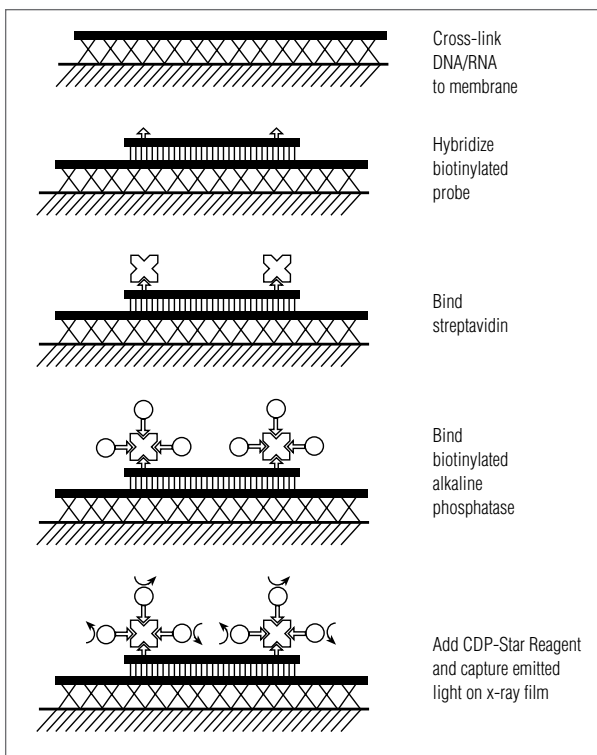
The enzyme alkaline phosphatase cleaves a phosphate group off the dioxetane substrate. The intermediate is unstable and decomposes, emitting light in the process.

Protocol Overview:

Steps for chemiluminescent detection using the Phototope-Star Detection Kit.

1. Biotinylated target DNA is immobilized on a membrane support or a biotinylated probe is hybridized to target DNA/RNA cross-linked on a membrane support.
2. Streptavidin is bound to the biotinylated DNA.
3. Biotinylated alkaline phosphatase is bound to streptavidin.
4. CDP-Star Reagent is reacted with the bound biotinylated alkaline phosphatase and the emitted light is captured on x-ray film.

Figure 2.



The Chemiluminescent Detection Process (diagram depicts chemiluminescent probing). This process is carried out through successive incubations with streptavidin, biotinylated alkaline phosphatase, and the CDP-Star Reagent. After the reagent is added, the membrane is briefly exposed to x-ray film to capture the image.

Additional Materials and Equipment for Detection

Solutions

Consult Appendix I for solution compositions and preparation procedures.

Detection Solution A (1X)

- 5% SDS, 125 mM NaCl
- 25 mM sodium phosphate
- pH 7.2

Detection Solution B

- 1:10 dilution of Detection Solution A

Detection Solution C (1X)

- 10 mM Tris-HCl
- 10 mM NaCl
- 1 mM $MgCl_2$
- pH 9.5 (Stock can be made 10X)

Materials and Equipment

- Hybridization bags or containers
- Platform shaker

Phototope Detection Protocol:

The following protocol will allow detection of biotinylated DNA on Southern and Northern blots, dot blots and DNA sequencing membranes (2,5,6). Detection of plaque lifts and colony hybridizations can be obtained using standard protocols. The kit is not yet recommended for *in situ* hybridization.

Note: In all steps during the detection procedures, it is important that the membrane is evenly covered with solution and that it floats freely in solution. If a hybridization bag is used, it must be free of trapped air to ensure maximum contact between the reagents and the membrane. Examples of the detection volumes necessary for a 10 cm x 10 cm membrane sealed in a hybridization bag are given. Volumes required for detections done in a container, or bag that is considerably larger than the membrane, may be greater. Be sure membrane stays completely wetted.

1. Wash Step – Detection Solution A

Add to the hybridization bag 0.1 ml of Detection Solution A per cm^2 membrane.

* Incubate for 5 minutes at room temperature with moderate shaking. Drain the solution before the next step.

Sample calculation:

$$100 \text{ cm}^2 \times 0.1 \text{ ml/cm}^2 = 10 \text{ ml}$$

2. Streptavidin incubation

Determine the necessary volume of *diluted* streptavidin based on using 0.05 ml of diluted streptavidin per cm² of membrane.* Prepare this volume by diluting the streptavidin stock solution 1:1000 with Detection Solution A. Incubate for 5 minutes at room temperature with moderate shaking. Drain the solution before next step.

Sample calculation:

$$100 \text{ cm}^2 \times 0.05 \text{ ml/cm}^2 = 5 \text{ ml}$$

Dilute 5 μ l of streptavidin stock solution to 5 ml with Detection Solution A.

3. Wash 2 times – Detection Solution B

Use 0.5 ml Detection Solution B per cm² of membrane.* Wash twice, 5 minutes each, at room temperature with moderate shaking. Drain and discard the solution after each wash.

Sample calculation:

$$100 \text{ cm}^2 \times 0.5 \text{ ml/cm}^2 = 50 \text{ ml each}$$

4. Biotinylated Alkaline Phosphatase incubation

Determine the necessary volume of *diluted* phosphatase based on using 0.05 ml of diluted phosphatase per cm² of membrane.* Prepare this volume by diluting the biotinylated alkaline phosphatase stock solution 1:1000 with Detection Solution A. Incubate for 5 minutes at room temperature with moderate shaking. Drain the solution before next step.

Sample calculation:

$$100 \text{ cm}^2 \times 0.05 \text{ ml/cm}^2 = 5 \text{ ml}$$

Dilute 5 μ l biotinylated alkaline phosphatase stock solution to 5 ml with Detection Solution A

5. Wash 1 time – Detection Solution A

Use 0.5 ml Detection Solution A per cm² of membrane.* Wash once for 5 minutes, at room temperature with moderate shaking. Drain and discard the solution.

Sample calculation:

$$100 \text{ cm}^2 \times 0.5 \text{ ml/cm}^2 = 50 \text{ ml}$$

6. Wash 2 times – Detection Solution C

Use 0.5 ml of 1X Detection Solution C per cm² of membrane.* Wash twice, 5 minutes each, at room temperature with moderate shaking. Drain and discard the solution after each wash.

Sample calculation:

$$100 \text{ cm}^2 \times 0.5 \text{ ml/cm}^2 = 50 \text{ ml each}$$

7. Detecting the DNA

The CDP-Star Reagent is supplied as a 25 mM stock solution and should only be diluted immediately before use. CDP-Star Dilution Buffer is supplied as a 25X stock solution.

CDP-Star Reagent stock solution can be diluted from 1:100 to 1:500* (CDP-Star Manufacturer recommends 1:100 dilution for all applications) depending on its intended use. For experiments requiring maximum signal intensity and signal longevity, dilute 1:100 with 1X CDP-Star Dilution Buffer. For maximum volume in experiments where sensitivity is not a problem, dilute up to 1:500 with 1X CDP-Star Dilution Buffer. Sufficient reagents are provided for detection on 4,000 cm² of membrane using the maximum sensitivity protocol or 20,000 cm² of membrane using the maximum volume protocol.

- a. Dilute the CDP-Star (25X) Dilution Buffer with Milli-Q™ water to a 1X concentration. 1X CDP-Star Dilution Buffer is stable at room temperature; therefore, large quantities can be prepared and stored for extended periods of time.
- b. Determine the amount of diluted CDP-Star Reagent needed and prepare this quantity immediately before use. Use 0.025 ml of diluted CDP-Star Reagent per cm² membrane. The diluted CDP-Star Reagent is prepared by diluting the 25 mM CDP-Star stock solution with the 1X CDP-Star Dilution Buffer.

Sample calculation:

100 cm² × 0.025 ml/cm² = 2.5 ml of diluted CDP-Star Reagent

For maximum sensitivity

Dilute 25 µl of 25 mM CDP-Star Reagent to 2.5 ml with 1X CDP-Star Dilution Buffer.

For maximum volume

Dilute 5 µl of 25 mM CDP-Star Reagent to 2.5 ml with 1X CDP-Star Dilution Buffer.

- c. Add the diluted CDP-Star Reagent to the hybridization bag. Incubate for 5 minutes at room temperature with moderate shaking.

8. Expose membrane

Open bag and drain as thoroughly as possible. Smooth out any wrinkles or air bubbles and reseal the bag. Clean the outside of the bag. It is important to have close, uniform contact between the film and the membrane to obtain sharp images, and be sure DNA side of membrane is toward the film. Expose the membrane to x-ray film for 15 seconds to 15 minutes or phospho-imager according to manufacturer's recommendations.

**Volumes required for detections done in a container, or bag that is considerably larger than the membrane, may be greater. Be sure membrane stays completely wetted.*

Frequently Asked Questions (FAQs):

Weak or Inconsistent Signal

How can I determine if the labeling of the probe is the problem?

Check the level of probe biotinylation by comparing to biotinylated control DNA and pre-biotinylated markers. If the labeled probe looks weak but the biotinylated control looks fine, the template DNA is most likely the problem.

To confirm probe quality check the starting amount of template and length of time of the random primer reaction. If the template DNA is less than 100 bp the efficiency of the random priming reaction will decrease.

Be sure the template is adequately purified. Also, confirm that the template is linear and completely heat denatured before biotinylation. If both the probe and biotinylated control look weak compared to pre-biotinylated markers, biotinylate again with fresh reagents.

To confirm probe specificity, dilute the target DNA and dot blot on a membrane. Hybridize with your probe followed by detection. Alternatively dot blot some of the plasmid DNA your probe was made from on a membrane, hybridize with your probe and detect.

How can I improve the priming reaction?

Primers and dNTPS can be added to the priming reaction at any time, i.e. before

denaturing the template DNA in boiling water, or they can be added after the template has been placed on ice. The Klenow, however, must be added only after the reaction tube has cooled as it will be inactivated during the boiling procedure.

The quantity of probe synthesized is dependent on the amount of template used and the length of time the reaction is allowed to run. Using smaller quantities of template DNA for a longer time will result in a higher ratio of labeled to unlabeled probe and will increase the efficiency of the labeling reaction. Running the labeling reaction overnight is acceptable to obtain high specificity probe from a very small amount of template DNA.

Adding carrier DNA or RNA during the ethanol precipitation step will reduce possible loss of the probe during precipitation. We recommend ethanol precipitation, spin columns or gel filtration to purify the probe.

What other reasons are there for a weak signal?

It could be that the transfer to the membrane was inadequate. Make sure there is good contact and no bubbles between membrane and the gel. Include a lane of pre-biotinylated markers in the gel to aid in troubleshooting. Inadequate cross-linking can also reduce the signal. Ensure the membrane is completely dry before cross-linking and the UV cross-linking was done according to manufacturer's protocols.

Another possibility is the hybridization reaction was inefficient. Increasing the probe concentration during hybridization will improve efficiency.

Adjusting the temperature, salt concentration, or hybridization time based on the percent homology between the probe DNA and the target nucleic acid can improve hybridization (2,6). Spot a dilution of the probe DNA on the membrane as an aid for troubleshooting. Low post-hybridization wash temperatures (below 68°C) will reduce stringency of hybridization. Formamide buffers can be used for lower stringency.

Finally, the problem may be related to the detection procedure. Make sure the membrane floats freely in detection reagents and that there are no bubbles. For exposure, drain well, smooth out bag to remove any air allowing for good contact with the film and re-expose for a longer period of time. Be sure the DNA side of membrane is toward the film.

Check the pH of 1X CDP-Star Dilution Buffer and adjust to pH 9.5. Optimal, steady light emission depends on the pH of substrate buffer. If the pH is too low it will quench the reaction, a high pH will allow for an instant reaction, a flash of light that is too difficult to record.

High or Uneven Background

How can I determine if detection procedure is the problem?

The concentrations of streptavidin and biotinylated alkaline phosphatase can affect the signal to background ratio. We recommend fresh dilutions of both reagents although we have found solutions up to a week old can still result in an adequate signal. Fresh solutions should be filter sterilized before storage to prevent contamination. We recommend Milli-Q water or water of equivalent purity for all reagents.

Incubation and wash times will affect the signal to background ratio. Increase the time or the volume in the washing steps to assure adequate washing, particularly after the addition of streptavidin. Thoroughly drain off the wash buffer before adding the CDP-Star Reagent.

If the membrane dries out during contact with the film, rinse the membrane with Detection Solution B from the NEB Phototope-Star Detection Kit and reapply CDP-Star Reagent. Seal the membrane in a bag before exposing to film.

Re-exposing the film for a shorter time may improve the background. Again, confirm that the DNA side of membrane is toward the film.

Background problems may be related to the concentration of the CDP-Star Reagent. The CDP-Star Reagent concentration may be too high or too low. Try different dilutions in the range of 1:100 to 1:500. Precipitation of the CDP-Star Reagent will affect the concentration. If a white precipitate is seen in the CDP-Star Reagent solution, warm to 37°C to resolubilize. For lower background use uncharged nylon membrane and UV cross-link instead of alkaline transfer. Nitrocellulose can quench chemiluminescence.

What else can I try?

Do not use powdered milk in the prehybridization buffer as the NEB Phototope-Star Detection Kit is sensitive to the biotin naturally present in milk. Pre-hybridize with denatured sperm DNA instead.

Check to make sure the template DNA is not too short (less than 100 bp) as this could lead to inefficient probe production. It may also be necessary to re-purify the template DNA. Excess probe will increase background.

The blotting procedure could lead to an uneven background. Make sure the blotting buffers are not contaminated. Filter sterilize or autoclave before storing. Always wear powder-free gloves and handle membranes carefully to avoid fingerprints, scrapes or damaged membranes. A static charge between the membrane and the film can result in dark lines.

The blotting procedure could lead to an uneven background. Make sure the blotting buffers are not contaminated. Filter sterilize or autoclave before storing. Always wear powder-free gloves and handle membranes carefully to avoid fingerprints, scrapes or damaged membranes. A static charge between the membrane and the film can result in dark lines.

General

Can you recommend a hybridization or detection bag?

Use bags made for the specific purpose of hybridization. They are available from suppliers of products for molecular biology. Use of Zip-Lock type bags is not recommended. "Seal-a-Meal" bags have been used successfully. Plastic containers of most any type, however, can be used.

Can I store the NEB Phototope Detection Kits at minus 20°C?

The NEB Phototope Detection Kit should be stored at 4°C. If the kit is frozen, the biotinylated alkaline phosphatase is sensitive to freezing. We recommend replacing the biotinylated alkaline phosphatase.

How sensitive is Chemiluminescent detection?

Chemiluminescent detection is more sensitive than colorimetric detection. However, for single copy genes from large genomes, chemiluminescent detection may not be as sensitive as radioactive detection.

Appendix I: Solution Compositions and Preparation

All solutions should be made with Milli-Q water, or water of equivalent purity.

Detection Solution A:

5% SDS, 25 mM Sodium Phosphate, pH 7.2 (17 mM Na_2HPO_4 , 8 mM NaH_2PO_4), 125 mM NaCl

Dissolve 7.3 g NaCl, 2.4 g Na_2HPO_4 (dibasic), 1.0 g NaH_2PO_4 (monobasic), and 50 g SDS in 800 ml water. Adjust the volume to 1 liter.

Detection Solution B:

0.5% SDS, 2.5 Sodium Phosphate (1.7 mM Na_2HPO_4 , 0.8 mM NaH_2PO_4), 12.5 mM NaCl

Dilute Detection Solution A 1 to 10 in water.

Detection Solution C (10X):

100 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl_2 , pH 9.5

Dissolve 12 g Tris base, 5.8 g NaCl, 2 g MgCl_2 in 800 ml water. Adjust the pH to 9.5 with HCl. Adjust volume to 1 liter. Use at 1X.

Filter sterilize and store at room temperature. A precipitate may form if stored at 4°C.

Appendix II: Membrane Recommendations

Phototope® Chemiluminescent Kits were developed using Millipore membranes. For most applications, other nylon membranes with a neutral or slight positive charge will work equally well. Please contact the suppliers directly to order the membrane of your choice, and membrane questions should be addressed with the supplier of the membrane. Nitrocellulose membranes are not recommended for Phototope-Star Detection Kit applications.

Appendix III: Protocol for Stripping Membranes

After detection with one probe is complete, the membrane can be stripped and washed to remove the probe. Hybridization with another probe can then be performed beginning with the prehybridization step.

Stripping Southern:

1. Rinse the membrane in water
2. Incubate in 0.4 N NaOH, 0.1% SDS at 80°C for 30 minutes.
3. Rinse membrane in 0.2 M Tris-HCl, 0.1X SSC at 25°C for 30 minutes.
4. The membrane can now be reprobed.

Stripping Northern:

1. Rinse the membrane in water.
2. Incubate in a large volume (~500 ml) of 2 mM EDTA, 0.1% SDS at 80°C for 15 minutes.
3. Rinse membrane in 2X SSC at room temperature.
4. The membrane can now be reprobed.

Note: Incubating for more than half an hour (overstripping) may adversely affect the target DNA/RNA bound to the membrane.

Storing the membrane

The membrane can be stored in a small volume of 1X SSC in a sealed bag at room temperature for the short term. For extended storage, freeze at -20°C. Do NOT allow the membrane to dry out.

References:

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2. Perry-O'Keefe, H. and Kissinger, C. M (1989). In F. M. Ausubel, R. Brent, R. E. Kingston, D. D. More, J. G. Seidman, J. A. Smith and K. Struhl (Eds.), *Current Protocols in Molecular Biology* (pp. 3.19.1–3.19.8). New York: Wiley-Interscience.
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The NEBlot Phototope[®] and NEB Phototope[®]-Star Detection Kits:

The NEBlot Phototope[®] and NEB Phototope[®]-Star Detection Kits are designed for the non-radioactive labeling and detection of nucleic acids. These kits offer the investigator the opportunity to replace radioactivity with chemiluminescence, a fast, safe, and economical alternative. The range of applications amenable to Phototope detection includes Southern and Northern blots, dot blots, and DNA sequencing membranes. Detection of plaque lifts and colony hybridizations can be obtained using standard protocols. The kit has not been tested for *in situ* hybridization.

Ordering Information

PRODUCT	NEB #	SIZE
Phototope® -Star Detection Kit	N7020S	
KIT COMPONENTS SOLD SEPARATELY		
CDP-Star Reagent	N7001S	2.5 ml
Streptavidin	N7021S	1.0 mg
Biotinylated Alkaline Phosphatase	N7022S	0.5 mg
CDP-Star (25X) Dilution Buffer	B7011S	25 ml
COMPANION PRODUCTS		
NEBlot® Phototope® Kit	N7550S	

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USA

New England Biolabs, Inc.
240 County Road
Ipswich, MA 01938-2723
Telephone: (978) 927-5054
Toll Free: (USA Orders) 1-800-632-5227
Toll Free: (USA Tech) 1-800-632-7799
Fax: (978) 921-1350
e-mail: info@neb.com
www.neb.com

Canada

New England Biolabs, Ltd.
Telephone: (905) 837-2234
Toll Free: 1-800-387-1095
Fax: (905) 837-2994
Fax Toll Free: 1-800-563-3789
e-mail: info@ca.neb.com

China, People's Republic

New England Biolabs (Beijing), Ltd.
Telephone: 010-82378265/82378266
Fax: 010-82378262
e-mail: info@neb-china.com

France

New England Biolabs France
Free Call: 0800/100 632
Free Fax: 0800/100 610
e-mail: info@fr.neb.com

Germany

New England Biolabs GmbH
Telephone: +49/(0)69/305 23140
Free Call: 0800/246 5227 (Germany)
Fax +49/(0)69/305 23149
Free Fax: 0800/246 5229 (Germany)
e-mail: info@de.neb.com

Japan

New England Biolabs Japan, Inc.
Telephone: +81 (0)3 5669 6191
Fax +81 (0)3 5669 6192
e-mail: info@neb-japan.com

United Kingdom

New England Biolabs (UK), Ltd.
Telephone: (01462) 420616
Call Free: 0800 318486
Fax: (01462) 421057
Fax Free: 0800 435682
e-mail: info@uk.neb.com