CHEMILUMINESCENT DNA DETECTION KIT

Product Information

Product No. **LUM-1**
Technical Bulletin No. MB-255

TECHNICAL BULLETIN

April 1998

The Chemiluminescent DNA Detection Kit is a complete system for the detection of biotin or alkaline phosphatase labeled probes in Southern and Northern blots. Typically, detection of DNA and RNA has been performed using ^{32}P and ^{35}S labeled probes. The sensitivity, speed and versatility of this chemiluminescent system eliminate the need to use hazardous radioactive methodology. All components of the kit have been optimized for maximum signal intensity and low background.

This procedure may be used to detect a nucleic acid probe labeled with biotin (using photoreactive biotin (Product No. A1935), Nick Translation Kit (NICK-1) or incorporation of biotinylated deoxynucleotides in a random primer reaction) or alkaline phosphatase. Biotinylated probes are detected by utilization of a streptavidin-alkaline phosphatase conjugate followed by reaction with CSPD substrate. Alkaline phosphatase labeled probes are detected directly by reaction with CSPD substrate.

The CSPD substrate is a direct chemiluminescent substrate for alkaline phosphatase. Upon enzymatic dephosphorylation, CSPD decomposes, releasing a constant emission of light with a maximum at 477 nm. The results can be conveniently imaged on x-ray film with exposure times of 5-120 minutes. The light emission persists for days allowing for multiple exposures. Additionally, the membranes can be easily stripped and reprobed. The chemiluminescent kit can also be used for the detection of DNA in colony hybridizations and plaque lifts.

Reagents Provided
Sufficient for fifty 10 cm X 10 cm blots

- I-Block Reagent, Product No. B7167 — 30 g
- CSPD Substrate, Product No. C6579 — 2.5 ml
  100X concentrate, 25 mM
- Diethanolamine, Product No. D3679 — 120 ml
  If material solidifies during storage, warm at 37-65°C to melt.
- Streptavidin-Alkaline Phosphatase Conjugate, Product No. S7671 — 200 µl
  Supplied in a buffered glycerol solution. Dilute 1:5,000 prior to use as described in the protocol. Store below 0°C.

Reagents and Equipment Required but Not Provided
(Sigma product numbers have been provided where appropriate)

Biotinylated or alkaline phosphatase labeled oligonucleotide probe
Nylon membrane (Product No. N1389)
Heat stable hybridization bags
0.5 M Sodium phosphate, dibasic, pH 7.2
20% Sodium dodecyl sulfate (SDS)
10X Phosphate buffered saline (PBS) (Product No. P3813)
20X Saline-sodium citrate (SSC) (Product No. S0902)
1 M Magnesium chloride (Product No. M1028)
Reagents required for use with alkaline phosphatase labeled probes:
Dextran sulfate (Product No. D8906)
Triton X-100 (Product No. T8787)
Trizma® Base (Product No. T8524)
Sodium chloride (Product No. S3014)

Precautions
Sigma's Chemiluminescent DNA Detection Kit is for laboratory use only. Not for drug, household or other uses. Kit contains components which are hazardous. Warning statements are included on the label or in the components section of this bulletin where applicable.

Storage
On receipt, store the streptavidin-alkaline phosphatase conjugate below 0°C. Store CSPD substrate at 2-8°C. Store I-block reagent and diethanolamine at room temperature.

Preparation Instructions
Prepare the reagents for the specific method to be used.

0.25 M sodium phosphate, dibasic, pH 7.2 for probe hybridization
Hybridization buffer for biotinylated probes: 1 mM EDTA, 7% SDS, 0.25 M sodium phosphate, dibasic, pH 7.2
Note: 5% Dextran sulfate may be added for long DNA probes
Wash buffers I, II and III for biotinylated probes:
Wash buffer I: 2X SSC, 1% SDS
Wash buffer II (for oligonucleotide probes): 1X SSC, 1% SDS
Wash buffer II (for long DNA probes): 0.1X SSC, 1% SDS
Wash buffer III: 1X SSC

Hybridization buffer for alkaline phosphatase labeled probes: 7% SDS, 0.25 M sodium phosphate, dibasic, pH 7.2, 1.0% I-Block reagent
Wash buffers I, II, III and IV for alkaline phosphatase labeled probes:
Wash buffer I: 5X SSC, 1% SDS
Wash buffer II: 1X SSC, 1% SDS
Wash buffer III: 1% Triton X-100, 125 mM NaCl, 50 mM Tris, pH 8.0
Wash buffer IV: 1X SSC

Blocking buffer for chemiluminescent detection: 0.2% I-Block reagent, 1X PBS, 0.5% SDS. Cool to room temperature before use.
Detection wash buffer: 1X PBS, 0.5% SDS
Assay buffer: 0.1 M diethanolamine, 1 mM magnesium chloride
Chemiluminescent substrate solution: 0.25 mM CSPD (Dilute 25 mM CSPD 1:100 in assay buffer)
Note: Sodium azide (Product No. S8032) (0.02% w/v) may be added to solutions if short term storage is anticipated. However, the use of freshly prepared solutions is highly recommended.

Procedures
Note: All incubations are at room temperature unless otherwise noted. Never touch membrane with ungloved hands and avoid excess pressure on filters (handle only at edges). Mishandled membranes exhibit high background signals.

A. Biotinylated Probe Hybridization

Oligonucleotide Probe Hybridization

1. Wet the membrane in 0.25 M sodium phosphate, dibasic, pH 7.2.

2. Prehybridize the membrane in hybridization buffer for 1 hour at 55°C or at an appropriate hybridization temperature. Drain the buffer.

3. Dilute the heat-denatured biotinylated probe to 0.1-5.0 pmol/ml in fresh hybridization buffer and add to the membrane (10-100 µl/cm^2). Incubate for 2 hours at the appropriate temperature.

4. Wash the membrane twice for 5 minutes each in wash buffer I (1 ml/cm^2). Wash twice for 15 minutes each at the hybridization temperature in wash buffer II for oligonucleotide probes. Wash twice for 5 minutes each in wash buffer III.

5. Proceed to the chemiluminescent detection of biotinylated DNA (Section C).

Long DNA Probe Hybridization

NOTE: Long DNA probes include those that have been prepared by nick translation and random primer labeling.

1. Wet the membrane in 0.25 M sodium phosphate, dibasic, pH 7.2.
2. Prehybridize the membrane in hybridization buffer for 1 hour at 65°C. Drain the buffer.

3. Dilute the heat denatured, biotinylated probe to 10-100 ng/ml in fresh hybridization buffer and add to membrane (10-100 μl/cm^2). Incubate overnight at 65°C.

4. Wash the membrane two times for 5 minutes each in wash buffer I (1 ml/cm^2). Wash two times for 15 minutes each at 65°C in wash buffer II for long DNA probes. Wash two times for 5 minutes each in wash buffer III.

5. Proceed to the chemiluminescent detection of biotinylated DNA (Section C).

B. Alkaline Phosphatase Labeled Probe Hybridization

1. Prehybridize the membrane for 30 minutes in hybridization buffer (10-100 μl/cm^2) using a temperature that is optimized for the probe.

2. Dilute the alkaline phosphatase labeled probe to 0.25-1.0 nM in fresh hybridization buffer (10-100 μl/cm^2) and incubate for 30 minutes at the appropriate temperature.

3. Wash the membrane four times for 5 minutes each at 45°C in wash buffer I (1 ml/cm^2). Wash two times for 15 minutes each at the appropriate hybridization temperature in wash buffer II. Briefly rinse the membrane once in wash buffer III followed by a 20 minute wash in wash buffer III.

4. Wash twice for 1 minute each in wash buffer IV.

5. Proceed to the chemiluminescent detection steps (Section C, steps 6-10).

C. Chemiluminescent Detection of Biotinylated DNA on Nylon Membrane

1. Wash the membrane twice for 5 minutes each in blocking buffer (0.5 ml/cm^2)

2. Incubate the membrane for 10 minutes in blocking buffer (1 ml/cm^2)

3. Dilute the streptavidin-alkaline phosphatase conjugate 1:5000 in blocking buffer (2.0 μl streptavidin-AP conjugate in 10 ml blocking buffer per 100 cm^2 membrane, 0.1 ml/cm^2). Incubate the membrane for 20 minutes with the conjugate solution with constant agitation.

4. Wash once for 5 minutes in blocking buffer (0.5 ml/cm^2).

5. Wash three times for 5 minutes each in detection wash buffer (1 ml/cm^2).

6. Wash twice for 2 minutes each in assay buffer (0.5 ml/cm^2).

7. Add the chemiluminescent substrate solution to the membrane (5 ml/100 cm^2).

8. Agitate slowly for 5 minutes.

9. Discard the excess substrate solution, but do not allow the membrane to dry.

10. Seal the membrane in a plastic bag (hybridization bag, plastic wrap, etc.) and proceed to Section D.

D. Film Exposure and Development

1. Place the sealed membrane in direct contact with Kodak XAR autoradiography film.

2. Expose the film for 5 to 30 minutes. This will help to determine the optimal exposure time for the specific application.

3. Develop the autoradiographic film following standard protocol.

E. Stripping and Reprobing Procedures

It is possible to remove a probe and re-hybridize a membrane with a different probe if the membrane has not dried out.

1. Wash the membrane twice for 20 minutes each in 0.1X SSC, 1.0% SDS at 95°C.

2. Wash the membrane twice for 5 minutes each in 1X SSC and air dry.

3. The membrane is now ready to be re-hybridized with a new probe. Successful removal of the biotinylated probes and streptavidin-alkaline phosphatase conjugate can be confirmed by checking for chemiluminescent detection (Section C, steps 6-10).

Results

Troubleshooting the procedure

Since CSPD provides extremely sensitive detection of alkaline phosphatase labeled probes, only molecular biology grade water and reagents should be used.

For Low Sensitivity:

1. All buffers should be prepared fresh daily for best results.

2 To detect low levels of DNA, lengthen the film exposure time until the desired band is visible.

3. Increase the incubation time for hybridization of probe to target to overnight and/or extend the incubation time of the streptavidin-alkaline phosphatase conjugate to 60 minutes.

4. Increase the concentration of labeled DNA and/or streptavidin-alkaline phosphatase conjugate. However, this may contribute to an increase in nonspecific binding.

5. Confirm the probe is effectively labeled and denatured prior to use. This can be accomplished by spotting serial dilutions on a membrane and detecting using chemiluminescence.

For High Background:

1. Bacterial contamination may result in distorted images. Make sure all buffers are free of contamination prior to use and the blot, blotting paper, and hybridization bags are clean. Gloves should be worn at all times during the procedure.

2. Decrease the film exposure time until the desired resolution is achieved.

3. If the background signal is even across the membrane obscuring the target signal, incubate the blot in blocking buffer overnight at 2-8°C or increase the number of wash steps after the strepavidin-alkaline phosphatase conjugate incubation.

4. To remove nonspecific binding of the strepavidin-alkaline phosphatase conjugate, increase the dilution to 1:10,000 to 1:15,000 and centrifuge to remove any particulate matter.

5. To remove nonspecific binding of the DNA probe, reduce the probe concentration or increase the duration of the final two hybridization wash steps.

6. If the background signal is spotty, purify the probe by ethanol precipitation.

References

1. Allefs, J.J.H.M., *et al.*, Nucl. Acids Res. **18**: 3099-3100 (1900)
2. Bronstein, I.,*et al.*, Anal. Biochem. **180**: 95-98 (1989)
3. Bronstein, I., and McGrath, P., Nature **338**: 599-600 (1989)
4. Bronstein, I., and Voyta, J.C., Clin. Chem. **35**: 1856-1857 (1989)
5. Bronstein,I., *et al.*, BioTechniques **8**: 310-314 (1990)
6. Holtke, H.J., *et al.*, Fresenius Z. Anal. Chem. **330**: 377-378 (1988)
7. Jablonski, E.,*et al.,* Nucl. Acid Res. **14**: 6115-6128 (1986)
8. Kreike, C.M.,*et al.*, Plant Mol. Biol. Reporter **8** (3): 172-179 (1990)
9. Kricka, L.J. (Ed.) Nonisotopic Probing, Blotting, and Sequencing, Second edition, Academic Press, p. 145-164, (1995). (Product No. Z36,402-9)
10. Langer, P.R., *et al.*, PNAS (USA) **78**: 6633-6637 (1981)
11. Lanzillo, J.J., BioTechniques **8**: 620-622 (1990)
12. Lanzillo, J.J., Anal. Biochem. **194**: 45-53 (1991)
13. Leary, J.J., *et al.*, PNAS (USA) **80**: 4045-4049 (1983)
14. Southern, E.M., J. Mol. Biol. **98**: 503-517 (1975)