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Catalog No. 54-30-00

INTRODUCTION

The DNADetector™ HRP Chemiluminescent Blotting Kit is designed for the hybridization and chemiluminescent detection of biotinylated DNA probes. Applications for detecting DNA through hybridization include Southern blotting, bacterial colony hybridization, plaque hybridization and dot blotting.

Nucleic acids immobilized on a solid support such as nylon membrane are hybridized with biotinylated DNA. The membrane is washed to remove excess probe and blocked to prevent non-specific binding. The membrane is then incubated with peroxidase-labeled streptavidin (HRP-SA), which binds biotin molecules with very high avidity. The membrane is washed again to remove excess HRP-SA, then incubated with LumiGLO Chemiluminescent Substrate. Exposure to X-ray film produces a permanent record of chemiluminescent emissions.

Chemiluminescent detection using LumiGLO substrate generates five to ten times more sensitivity than colorimetric methods and permanent results of publication quality. Chemiluminescent detection also allows multiple stripping of probe and reprobing of target bound to nylon membrane. Blots developed using this kit can also be detected using chemiluminescent imaging systems.

Colorimetric detection can be performed with this kit in conjunction with TMB Membrane Substrate (Catalog No. 50-77-03) to save the time and expense of using film and related equipment. Colorimetric detection is especially useful for control experiments because variability due to the film exposure process is eliminated.

MATERIALS AND EQUIPMENT

Kit Components	Product Code	Volume
Formamide Hybridization Buffer	50-86-09	120 mL
Detector Block Solution (5X)	71-83-02	240 mL
Detector Block Powder	72-01-03	10 g
Peroxidase-labeled Streptavidin (HRP-SA)	474-3003	1.0 mL
Biotin Wash Solution Concentrate (10X)	50-63-05	3 x 100 mL
LumiGLO Peroxidase Chemiluminescent Substrate Solution A	50-59-00	120 mL
LumiGLO Peroxidase Chemiluminescent Substrate Solution B	50-60-00	120 mL

Sufficient reagents are provided to test approximately 2000 cm^2 of membrane (approximately 20, 10-cm x 10-cm blots) when recommended volumes are used. Reagents are stable for a minimum of one year when stored at 2 - 8°C.

For optimal performance, KPL recommends storing Formamide Hybridization Buffer at 2 - 8°C; warm the solution (37 - 50°C) and aliquot into DNase/RNase free tubes. Do not store Formamide Hybridization Buffer at -20°C. Prolonged storage of this buffer at -20°C may result in decreased sensitivity.

PRODUCT SAFETY AND HANDLING

See MSDS (Material Safety Data Sheet) for this product.

REQUIRED SUPPLIES AND EQUIPMENT NOT INCLUDED

- Biotin-Labeled Nucleic Acid Probe
- Herring Sperm DNA (Catalog No. 60-00-14)
- Ethidium Bromide
- 20X SSPE
- 20X SSC (Catalog No. 50-86-05)
- Gloves
- Micropipettors and sterile tips
- UV Transilluminator or vacuum oven
- Capillary transfer apparatus
- Waterbath or hybridization oven
- Oven or heat block at 95°C
- X-ray film (example: Kodak BioMax™ Light) and film cassettes or chemiluminescent imaging system
- Nylon, nitrocellulose or PVDF membrane (Pall Biodyne® B, Catalog No. 60-00-50 or MSI MagnaGraph for most applications)
- Heat-sealed hybridization bags (Catalog No. 60-00-51) or hybridization bottles.
- Molecular biology grade water (DNase/RNase free)

NOTE ON…WARNINGS AND PRECAUTIONS

⇒ Read ALL instructions thoroughly before using the kit.

⇒ Always wear protective gloves and a lab coat.

⇒ Ultraviolet light is harmful to skin and eyes. Shield skin and eyes from UV rays using UV-resistant glasses and protective clothing.

⇒ Formamide is a suspected teratogen and its use should be restricted to a fumehood.

SOUTHERN BLOTTING PROTOCOL

A commonly used technique by molecular biologists, Southern blotting involves the transfer and subsequent detection of electrophoretically separated DNA on membrane. Analysis of the immobilized DNA is facilitated by hybridization with an appropriately labeled nucleic acid probe, for which methods have been described earlier in this guide. Visualization of the target DNA can provide information regarding the quantity of a specific sequence as well as its size. This type of information serves numerous research goals such as gene identification, gene cloning, RFLP analysis, VNTR analysis and gene cloning.

The Detector HRP Chemiluminescent Blotting Kit was designed for the detection of plasmid and multiple-copy genomic Southern blots. Using LumiGLO Chemiluminescent Substrate, this kit enables detection of 0.3 pg plasmid DNA after just 15 minutes. Sensitivity after a 15 minute film exposure is equivalent to an overnight ^{32}P exposure.

While there are a variety of Southern blotting procedures, the following protocol is recommended when using the Detector HRP Chemiluminescent Blotting Kit to deliver the greatest sensitivity without background.

NOTE ON…PREPARATION OF BIOTINYLATED PROBES

⇒ Biotinylated probes may be prepared using the KPL Detector Random Primer DNA Biotinylation Kit (Catalog No. 60-01-00), Detector PCR DNA Biotinylation Kit (Catalog No. 60-01-01) or Detector RNA *in vitro* Transcription Biotinylation Kit (Catalog No. 60-01-02).

⇒ Quantitate your probe. The concentration of labeled probe should be determined by the quantitation procedure described in KPL's Biotinylation Kits. Excessive amounts of probe may result in non-specific signal while the addition of too little probe may result in insufficient signal.

Detector™ HRP Southern Blotting
At A Glance

Gel Electrophoresis

⇓

Alkaline Transfer

⇓

Prehybridization

1 hour

⇓

Hybridization

3 - 16 hours

⇓

0.5X SSPE

2 x 10 minutes

⇓

0.5X SSPE

2 x 10 minutes

⇓

2X SSPE wash

5 minutes

⇓

Detector Block

30 minutes

⇓

HRP-Streptavidin

20 minutes

⇓

Biotin Wash Solution

3 x 5 minutes

⇓

LumiGLO

1 minute

⇓

Film Exposure

1 minute - 2 hours

GEL ELECTROPHORESIS OF DNA

STEPS	CRITICAL POINTS
1. Digest target DNA with the restriction enzyme(s) of choice.	*High quality, contaminant-free target DNA is crucial to the success of hybridization experiments. DNA with A_{260}/A_{280} ratios >1.8 and A_{270}/A_{260} ratios = 0.8 should be used.* *The choice of enzymes is determined by the parameters of the experiment. Follow the manufacturer's recommendations for using restriction enzymes.*
2. Perform gel electrophoresis of DNA according to standard techniques. Include 0.5 µg/mL Ethidium bromide in the gel. Load 5 - 10 µg of genomic DNA for detection of single copy genes.	*The percentage of agarose, buffer-system and voltage during electrophoresis should be selected to provide optimal resolution of the samples.* ***Ethidium bromide is a powerful mutagen. Handle with extreme care!*** *Do not allow solutions containing ethidium bromide to contact skin or eyes.*
3. After electrophoresis, place the gel on a UV-transilluminator to view the fluorescent DNA sample. Photograph the gel next to a fluorescent ruler to facilitate determination of the molecular weight of the bands on the blot.	
4. **Immediately continue with the transfer.**	

ALKALINE TRANSFER OF DNA

Alkaline transfer is highly recommended for transfer of DNA onto positively charged nylon membrane. This method is more reproducible than high salt overnight transfers. It has also been shown to be the most efficient method; alkaline transfer occurs within 1 - 3 hours depending on the amount of DNA to be transferred.

STEPS	CRITICAL POINTS
1. Prepare 1 L of 5X SSC/10mM NaOH alkaline transfer buffer. Pre-treat the nylon membrane as recommended by the manufacturer.	*One Liter of transfer buffer is sufficient for the transfer of a 12 cm x 14 cm gel. Positively charged nylon is the preferred membrane for this application, specifically Pall Biodyne B Membrane.* *Always use clean forceps to handle membrane.*
2. If required, depurinate DNA by incubating the gel in two gel volumes of 0.25 N HCl for 10 minutes at room temperature with gentle agitation.	*Depurination is not necessary if the target of interest is <10kb. Continue to the denaturation step.*
3. Rinse the gel in molecular biology grade water and denature the DNA by incubating the gel in two gel volumes of 0.5 N NaOH/1.5 M NaCl for 30 minutes.	
4. Equilibrate the gel with 2 gel volumes of alkaline transfer buffer for 2 washes 10 minutes each at room temperature.	
5. Assemble a capillary transfer according to standard techniques using the transfer buffer as the solvent.	*To avoid excessive compression of the gel matrix, the weight placed on top of the transfer should not exceed 2 - 3 g/cm^2 of gel.*
6. Transfer time will depend on the amount of DNA loaded on the gel: >5 µg of DNA, transfer 3 hours; <5 µg of DNA, transfer for 2 hours; <100 ng, transfer for 1 hour.	

(ALKALINE TRANSFER OF DNA CONTINUED FROM PAGE 8)

STEPS	CRITICAL POINTS
7. After transfer, rinse the membrane for 5 minutes in 5X SSC. Place membrane on filter paper 2 - 4 minutes and fix the DNA to the membrane using a UV crosslinker or vacuum oven according to manufacturer's instructions. 8. Store membranes between two pieces of blotting paper and seal in a hybridization bag. Store bag in a cool and dry place.	*Despite claims by other manufacturers, KPL recommends fixing of nucleic acids to positively charged membranes by cross-linking or baking to achieve greatest sensitivity.*

PREHYBRIDIZATION & HYBRIDIZATION OF SOUTHERN BLOT

STEPS	CRITICAL POINTS
1. Place the Formamide Hybridization Buffer bottle in a water bath or incubator at 37°C to solubilize the SDS that has precipitated.	
2. Determine the amount of Prehybridization/Hybridization Buffer that is needed for your particular blot. A volume of 0.06 mL/cm^2 of membrane is recommended. Use the guidelines listed at the right.	*Use 0.06 mL of Formamide Hybridization Buffer per cm^2 membrane. (For example: a 10 cm x 10 cm membrane = 100 cm^2 = 6 mL buffer). The volume of buffer may be adjusted depending on the size of the membrane and the vessel used for hybridization. Hybridization bottles are recommended; alternatively, heat sealed bags may also be used. A minimum volume of 3 mL is required when using a 4 cm diameter x 14 cm long hybridization bottle. If less than 3 mL is needed, a hybridization bag is recommended.* *For convenience, 10 mL of Hybridization Buffer regardless of blot size may be used. Additional Formamide Hybridization Buffer is available for individual purchase.*
3. Prepare prehybridization solution by adding sheared and denatured herring or salmon sperm DNA to a final concentration of 200 µg per mL of Formamide Hybridization Buffer that is used. If using KPL's Herring Sperm DNA, add 10 µL per mL of Hybridization Buffer.	*If using non-denatured blocking DNA, heat denature for 5 minutes, cool quickly on ice and add to prehybridization solution.*
4. Place the membrane in a hybridization bottle with the DNA facing toward the middle of the bottle or in a hybridization bag, and add the prehybridization solution.	*If you seal membranes in heat-sealed plastic hybridization bags for hybridization, remove as much air and bubbles as possible before sealing the bag.* ***For best results, seal bag close to the edge of the membrane.*** *Do not allow membranes to stick together or to the sides of the hybridization bag.*

(PREHYBRIDIZATION & HYBRIDIZATION OF SOUTHERN BLOT CONTINUED FROM PAGE 10)

STEPS	CRITICAL POINTS
5. Incubate 1 hour at 42°C with constant agitation.	
6. Denature the DNA probe at 95°C for 10 minutes. Immediately place on ice.	*Do not denature probes by alkaline treatment.*
7. Add the probe to the Prehybridization Buffer at 50 ng per mL of Buffer.	*Pipette the probe directly into the buffer and swirl. Do not pipette directly on the blot.*
8. Incubate the membrane for 3 - 16 hours at 42°C with agitation.	*The desired hybridization temperature when using DNA probes is 42 °C.*
9. Prepare post-hybridization wash: 0.5X SSPE. Place one aliquot at room temperature. The other aliquot should be equilibrated to 50°C.	*Washes may be modified to contain different concentrations of SSPE and/or SDS to control stringency of the probe-target hybrid. Optimization for each individual probe is recommended.* *Equilibrate the 50°C solution at the elevated temperature at least 2 hours prior to use.*
10. Remove the membrane from the Hybridization buffer. Wash 2 x 10 minutes in a generous volume (at least 1 mL per cm^2 of membrane) 0.5X SSPE at room temperature.	*The Hybridization buffer with probe can be saved and reused. Save the buffer in a sterile conical tube at 2-8 °C. To reuse it, denature the solution at 68 °C for 10 minutes prior to hybridization. Do not boil.*
11. Wash with gentle agitation 2 x 10 minutes at the elevated temperature in the temperature equilibrated 0.5X SSPE wash.	*Generously cover the membrane with the wash solution.*
12. **Continue immediately with detection.**	*Never allow membrane to dry out during hybridization and detection.*

DETECTION OF SOUTHERN BLOT

STEPS	CRITICAL POINTS
1. Prepare enough 1X Detector Block blocking/diluent solution for the block step and for the HRP-SA conjugate dilution.	*Use at least 0.3 mL 1X blocking/diluent solution per cm^2 membrane (i.e. 10 cm x 10 cm = 100 cm^2 = 30 mL per incubation).* *See Buffer Preparation for preparation of 1X Detector Block.*
2. Incubate Southern blot with 1X Detector Block Solution for 30 minutes in a tray approximately the same size as the blot.	*All steps are to be carried out at room temperature with gentle agitation or rocking.* *Decrease the size of the container or increase the volume of the solution if the block is not free-flowing over the membrane.*
3. Dilute HRP-SA conjugate at 1/500 in fresh 1X Detector Block (60 µL conjugate + 30 mL fresh blocking solution). Mix well.	
4. Pour off the blocking/diluent solution (from step 2) from the membrane and add the diluted HRP-SA solution. Incubate for 20 minutes.	
5. Transfer membrane to a clean container. Wash the membrane in 1X Biotin Wash Solution. Perform 3 washes for 5 minutes each.	*Use 0.4 mL diluted wash solution per cm^2 of membrane (i.e. 10 cm x 10 cm membrane = 100 cm^2 = 40 mL wash solution per wash).* *See Buffer Preparation for instructions on preparation of 1X Biotin Wash Solution.*
6. Prepare enough LumiGLO Chemiluminescent Substrate to completely immerse the membrane by mixing equal volumes of Solutions A and B.	*Prepared LumiGLO Substrate is stable for 24 hours when stored at 4 ℃.*
7. Incubate membrane for 1 minute in LumiGLO and touch the corner to a clean piece of filter paper to absorb excess solution. Place membrane in a hybridization bag or between sheet protectors and expose to X-ray film for an initial exposure of 10 minutes. Adjust exposure time for optimal signal-to-noise ratio.	*If detecting genomic DNA, the initial exposure time should be increased to 30 - 60 minutes.*

(DETECTION OF SOUTHERN BLOT CONTINUED FROM PAGE 13)

STEPS	CRITICAL POINTS
8. Develop film either manually or by using a mechanical processor.	

STRIPPING AND RE-PROBING BLOTS

Membranes hybridized with biotinylated probes can be stripped and re-probed after detection with LumiGLO Chemiluminescent Substrate provided the membrane is never allowed to dry prior to stripping. That is, membranes should be stored in a covered container in 1X SSPE until such time as stripping and re-probing is desired.

SOUTHERN BLOTS

STRIPPING A DNA PROBE

1. Wash membrane in 1X Biotin Wash Solution for 5 minutes at room temperature.
2. Incubate membrane in 0.2 N NaOH/0.1% SDS (pre-warmed to 55°C) for 20 minutes at 55°C.
3. Rinse the membrane 2 times for 5 minutes each in 2X SSC.
4. Reprobe immediately or store the stripped membrane dry at room temperature or 4°C until ready to use.

BACTERIAL COLONY LIFTS

This protocol can be used to identify specific clones in a genome-sized library or to identify subclones made from a larger sequence. Bacterial cultures containing plasmids or cosmids are immobilized or fixed to nylon membrane and hybridized with a biotinylated probe to detect the colonies that harbor the plasmid or cosmid containing the target insert.

1. Plate cells on selective media (i.e. LB Media + ampicillin, 50 µg/mL) and incubate inverted overnight at 37°C.
2. Pre-cool the plates at 4°C for 30 minutes. Carefully lay a 1.2 micron pore size nylon membrane onto the agar plate containing the colonies. Begin at one edge of the plate and lay the membrane down smoothly, avoiding bubbles. Using a sterile glass spreader, lightly smooth the membrane onto the surface of the plate.
3. Mark membrane with India ink in a distinctive asymmetrical pattern. Dipping a syringe needle into ink and punching the needle through the membrane down into the agar leaves distinct markings on both the replica-membrane and the plate that are easy to realign upon completion of the procedure.
4. Remove replica-membrane from the plate. Seal the master plate with parafilm and store at 4°C.
5. Place membrane, colony-side up, onto filter paper saturated with 0.5 M NaOH/1.5 M NaCl. Incubate 5 minutes.
6. Briefly blot the membrane on dry filter paper.
7. Place membrane, colony-side up, onto filter paper saturated with 1.5 M NaCl/0.5 M Tris-HCl, pH 7.4. Incubate for 5 minutes.
8. Briefly blot the membrane on dry filter paper.
9. UV crosslink DNA to the membrane according to manufacturers instructions. Alternatively, the membrane can be baked for 30 minutes at 80°C.
10. Hybridize as described on pages 10 - 12 and detect as described on pages 13 - 14.

NOTE ON…EXPOSURE TIMES FOR DETECTION OF COLONY LIFTS

⇒ It is recommended that various exposure times be taken when using the colony lift protocol. A short exposure will only show the positive colonies. With a longer exposure, negative colonies or non-specific binding (to endogenous proteins in *E.coli*) will be visualized. Exposure times of 1, 5 and 10 minutes are recommended.

PLAQUE LIFTS

Bacteriophage DNA fixed to the membrane can be hybridized with a biotinylated probe to identify recombinant phage containing the target.

1. Plate bacteriophage on a lawn of the appropriate host *E.coli* strain. Use NZY+ agarose plates for secondary or final screens. Incubate the plates for 14 to 18 hours at 37°C.
2. Chill plates for 1 hour at 4°C to increase the strength of the top agar.
3. Lay a 1.2 micron pore size nylon membrane onto the surface of the plate. Wait 5 minutes. Mark membrane with India ink in a distinctive asymmetrical pattern. Dipping a syringe needle into ink and punching the needle through the membrane down into the agar leaves distinct markings on both the membrane and the plate that are easy to realign upon completion of the procedure.
4. Remove membrane from the plate. Place membrane phage-side-up onto a piece of filter paper saturated with 0.5 M NaOH/1.5 M NaCl for 5 minutes. To avoid diffusion of the phage by over-wetting the filter paper, use only enough solution to evenly wet it.
5. Briefly blot membrane on dry filter paper.
6. Place membrane onto filter paper saturated with 1.0 M Tris (pH 8.0)/1.5 M NaCl to neutralize for 5 minutes.
7. Briefly blot membrane on dry filter paper.
8. Place membrane onto filter paper saturated with 2X SSC/0.2 M Tris (pH 7.5) for 2 minutes.
9. Fix DNA to the membrane by baking at 80°C for 30 minutes or by UV crosslinking.
10. Hybridize as described on pages 10 - 12 and detect as described on pages 13 - 14.

DOT BLOTS

For fixing samples of target DNA to nylon membranes to be hybridized with biotinylated probe. Dot blots provide a simple method for determining optimal hybridization conditions or for determining whether a probe will detect homologous sequence in the target sample. Detection in the range of 1 ng - 0.1 pg of DNA can be expected using this procedure.

1. Grid nylon membrane with a matrix of blocks measuring from 0.5 to 1.0 cm. If using a dot blot filter manifold, follow the manufacturer's instructions.
2. Wet nylon membrane with molecular biology grade water for 5 minutes, then transfer to 5X SSC for 5 minutes. Dry the membrane.
3. Denature target DNA by boiling for 5 minutes, then place on ice until use.
4. Prepare dilutions of the target sample in 1X TE using silanized tubes or 2X SSC/0.1% SDS using non-silanized tubes.
5. Spot 1 µL of each dilution onto the matrix so that the dots form a series. Label the matrix with a # 2 pencil so that the orientation of the dots are the DNA-side of the membrane can be identified.
6. Allow dots to dry for 10 minutes. UV-crosslink DNA to the damp membrane according to manufacturers' instructions or bake for 30 minutes at 80°C.
7. Hybridize as described on pages 10 - 12 and detect as described on page 13 - 14.

LumiGLO Chemiluminescent Substrate

⇒ LumiGLO can be used with nitrocellulose, nylon and PVDF membranes.

⇒ The LumiGLO reaction does not need to be protected from light.

⇒ For maximum signal, expose membrane to film immediately after incubation with LumiGLO. The reaction and film exposure are performed at room temperature. For most applications, exposures of one hour or less produce sufficient sensitivity.

⇒ LumiGLO is an extremely sensitive substrate. Insufficient washing of membranes or contamination of substrate with HRP will result in non-specific background. Following incubation with HRP-SA, it is important to transfer membranes to a clean container and wash thoroughly to remove excess enzyme and prevent background problems. Always use a clean container for the substrate incubation.

⇒ Do not allow LumiGLO to contact film. LumiGLO solution will cause dark spots to appear on the film.

⇒ Following detection with LumiGLO, membranes may be developed with chromogenic TMB Membrane Substrate (50-77-03). After film exposure, wash membrane in diluted wash solution for 2 minutes. Immerse membrane in TMB Membrane Substrate until desired endpoint is seen (2 - 10 minutes). Rinse membrane in molecular biology grade water for one minute to stop the reaction, then air dry.

LumiGLO Light Emission Over Time

Light emission begins immediately upon incubation with LumiGLO and declines gradually over a period of 1 - 2 hours. Diminished enzyme activity after one hour is due to exposure of the enzyme to the products of the substrate reaction.

TROUBLESHOOTING GUIDE FOR HRP DETECTION ON MEMBRANES

PROBLEM 1: HIGH BACKGROUND OVER THE ENTIRE BLOT

Possible Cause	Corrective Measure
• Over-exposed film	Shorten the exposure time to film.
• Excess probe in hybridization cocktail	Quantitate the probe and add only 50 ng per milliliter of hybridization buffer i.e. 500 ng in 10 mL of hybridization buffer.
• Membrane dried out at some point during the assay procedure	Use appropriately sized containers and enough of the solutions to make sure the membrane is immersed and moving freely at all times during the assay.
• Insufficient blocking	Make sure the Detector Block Powder is completely in solution. There should be no clumps of powder remaining in the solution.
• Excessive conjugate was added to the blot	Add the conjugate at 1/500, i.e. 100 µL of conjugate to 50 mL of diluent.
• Post-hybridization washes were not stringent enough to wash the probe off of the membrane	Increase the stringency of the washes by decreasing the salt concentration or elevating the wash temperature. Make sure the elevated wash temperature is equilibrated at the higher temperature prior to use.

PROBLEM 2: SPOTTY BACKGROUND, NOT ALL OVER THE BLOT

Possible Cause	Corrective Measure
• Particulate in solutions or dusty containers used	Make sure all solutions are homogeneous. If there is a precipitate in the solution warm it prior to use. Use only clean containers free of dust, lint and free of DNase or RNase activity.
• Substrate has come into contact with the X-ray film	Seal the membrane in a plastic sheet protector or hybridization bag prior to exposure to film.

PROBLEM 3: SMUDGES OR SPOTS ON FILM

Possible Cause	Corrective Measure
• Fingerprints or dirty forceps have come in contact with the blot	Wear gloves and use forceps when handling the membrane; rinse forceps after handling the membrane when it has been in the conjugate solution.

PROBLEM 4: SIGNAL APPEARING AS SCRATCHES ON FILM

Possible Cause	Corrective Measure
• The membrane was scratched or damaged	Do not use containers that have rough spots or burrs that might damage the membrane; do not use rusty razor blades or scissors to cut the membrane.
• Static electricity was exposed to the film	Do not wear gloves when handling the film because it can produce static electricity, resulting in the appearance of lightning bolts when developed.

PROBLEM 5: LANE SPECIFIC BACKGROUND OR SMEARS WITHIN GEL LANES

Possible Cause	Corrective Measure
• Nucleic acid has degraded	Use highly purified nucleic acids so that the DNases and RNases are removed and nucleic acid is in tact; avoid shearing the nucleic acid during isolation and purification.
• Non-specific hybridization	Increase the stringency of the post hybridization washes by increasing the temperature or decreasing the salt concentration of the buffer i.e. increase the wash temperature to 55°C – 60°C for DNA probes or 68°C for RNA probes.
• Non-specific hybridization	Make sure to include sheared, denatured Herring sperm DNA in the hybridization cocktail.
• Probe concentration is too high	Decrease the probe concentration in the hybridization cocktail.

PROBLEM 6: LOW SIGNAL

Possible Cause	Corrective Measure
• Probe was not denatured	Check the temperature of the heating apparatus or use a boiling water bath to denature the probe.

PROBLEM 6: LOW SIGNAL CONTINUED

Possible Cause	Corrective Measure
• Inefficient transfer	Verify the transfer of nucleic acids by viewing the gel and membrane under UV illumination. Make sure to denature DNA gels in two gel volumes of 0.5 N NaOH/1.5 M NaCl for 45 minutes prior to transfer, even when performing an alkaline transfer. If the nucleic acid has not completely transferred, increase the transfer time. (Note: For Northern blotting, all of the 28s ribosomal RNA may not transfer). Transfer times may need to be optimized. Transferring too long under alkaline conditions may cause the nucleic acids to transfer through the membrane ("blow through"). Follow the general guidelines in this manual.
• Hybridization conditions too stringent	Decrease the temperature of hybridization.
• Post-hybridization wash conditions are too stringent	Decrease the wash stringency by increasing the salt concentration and/or decreasing the temperature of the washes.
• Insufficient biotinylated probe added to the hybridization	Make sure to quantitate your probe using the supplied Quantitation Standard. 50 ng per mL of Hybridization Cocktail should be added. If the background is low, 100 ng per mL may be added.
• Degradation of probe	Check the integrity of the probe by running it on an agarose gel. RNA and PCR probes should be a single band of distinct size. The random primed probes will appear as a smear with the majority of the probe ~ 200 - 300 bp.

PROBLEM 7: DIFFUSE SIGNAL

Possible Cause	Corrective Measure
• Excessive space between the membrane and the film	Make sure the film cassette is closed tightly or place a heavy book on top of it to ensure the membrane is tightly pressed against the film.
• The DNA side of the membrane faces away from the film	Make sure the DNA side of the membrane is facing the film.

PROBLEM 8: CIRCULAR PATTERNS OR WEAK SIGNAL IN SPECIFIC PLACES ON THE BLOT

Possible Cause	Corrective Measure
• Air bubbles were trapped between the membrane and gel during the transfer	Carefully set up the transfers so that all air bubbles are removed prior to the transfer.

PROBLEM 9: GHOST IMAGES I.E. FAINT SIGNAL DEVELOPMENT NEXT TO ACTUAL BANDS

Possible Cause	Corrective Measure
• The film or membrane shifted during the film exposure	Avoid repositioning the film or membrane once they come in contact with one another.

BUFFER PREPARATION

Sufficient reagents are provided in the Detector HRP Chemiluminescent Blotting Kit when volumes are used as indicated. If desired, increased working volumes may be used; however, additional reagents will be necessary. For convenience, buffer recipes and protocols are provided below.

1X DETECTOR BLOCK SOLUTION – TO BE PREPARED FRESH DAILY

STEPS	CRITICAL POINTS
1. Based on the total desired 1X Detector Block volume, weigh out 1.0% w/v Detector Block Powder for detection with HRP and LumiGLO.	*If the block solution is not prepared daily, sensitivity could be reduced and background will increase.*
2. Place the Detector Block Powder in a flat-bottom, screw cap container and add molecular biology grade water to a volume equivalent to 4/5 of the total desired 1X Detector Block volume. Shake the container vigorously until the powder is fully solubilized.	*Conical tubes are not recommended in the preparation of 1X Detector Block. If used, the solution may be vortexed to remove any packed Detector Block Powder from the bottom of the tube.*
3. Once the powder is in solution, dilute the solution with 1/5 v/v 5X Detector Block Solution. Example, for 50 mL of 1X Detector Block: Detector Block Powder 0.5 g Molecular Biology Grade H_20 40 mL 5X Detector Block Solution 10 mL	*Insure that all Detector Block Powder is in solution to avoid speckling patterns on the blot or insufficient blocking. The amount of powder used can be increased to decrease background. However, excessive Detector Block Powder may reduce sensitivity.*

1X BIOTIN WASH SOLUTION

STEPS
1. Dilute 10X Biotin Wash Solution Concentrate by diluting 1/10 in molecular biology grade water, i.e., 1 part Biotin Wash Solution Concentrate + 9 parts H_2O. Mix well.

20X SSC

STEPS
1. Mix together the following: 3.0 M NaCl 300 mM Sodium Citrate, pH 7.0
2. Sterile filter or autoclave solution

20X SSPE

STEPS

1. Mix together the following:
   3.0 M NaCl
   200 mM NaH2PO4
   20 mM EDTA, pH 7.4
2. Sterile filter or autoclave solution.

LB Media (for bacterial colony lifts)

STEPS

1. Mix together the following:
   5 g/L bacto-yeast extract
   10 g/L bacto-tryptone
   10 g/L NaCl
2. pH to 7.0 with NaOH.
3. Autoclave

NZY Media (for plaque lifts)

STEPS

1. Mix together the following:
   5 g/L NaCl
   2 g/L $MgSO_4 \bullet 7H_20$
   5 g/L yeast extract
   10 g/L NZ amine (casein hydrolysate)
2. pH to 7.5 with NaOH.
3. Autoclave

NOTE ON…Preparing agar plates for colony/plaque lifts

⇒ For agar plates, add agar to 1.5%

⇒ For top-agarose, add agarose to 0.7%

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