# *KPL Detector™*

# *AP Chemiluminescent Blotting Kit*

| **Products** | **Catalog No.** |
|---|---|
| KPL AP Chemiluminescent Blotting Kit, *2000 $cm^2$ membrane* | 5910-0028 (54-30-01) |
| KPL AP Chemiluminescent Blotting Kit, *500 $cm^2$ membrane* | 5910-0029 (54-30-02) |

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## INTRODUCTION

The KPL Detector™ AP Chemiluminescent Blotting Kit is designed for the hybridization and chemiluminescent detection of biotinylated DNA or RNA probes on membranes. Applications for detecting biotinylated probes through hybridization include Northern, Southern and dot blotting. Given the sensitivity of this system, the KPL Detector AP Chemiluminescent Blotting Kit was specifically designed for the non-radioactive detection of single copy genes and low expressed transcripts.

Nucleic acids immobilized on a solid support such as nylon membrane are prehybridized, then hybridized with biotinylated DNA or RNA probes. The membrane is washed to remove non-specifically bound probe and blocked to prevent non-specific binding of conjugate or reporter protein. The membrane is then incubated with alkaline phosphatase-labeled streptavidin (AP-SA), which binds biotin molecules with very high avidity. The membrane is washed again to remove excess AP-SA, then incubated with CDP-Star® Chemiluminescent Substrate.

Chemiluminescent detection using CDP-Star substrate generates greater sensitivity and faster exposure time than ECL-like substrates. Exposure to X-ray film produces a permanent record of chemiluminescent emissions. Detection may also be accomplished using a chemiluminescent imaging system.

The KPL Detector™ AP Chemiluminescent Blotting Kit was developed and optimized with Pall Biodyne® B nylon membrane for remarkably high sensitivity and low background. Protocols for both Northern and Southern blotting applications are provided in this manual.

## MATERIALS AND EQUIPMENT

| | **5910-0028 (54-30-01) 2000 cm$^2$** | | **5910-0029 (54-30-02) 500 cm$^2$** | |
|---|---|---|---|---|
| **Kit Components** | **Cat. No.** | **Volume** | **Cat. No.** | **Volume** |
| KPL Formamide Hybridization Buffer | 5910-0001 (50-86-12) | 200 mL | 5960-0024 (50-86-11) | 50 mL |
| KPL 5X Detector Block | 5920-0005 (71-83-02) | 2x240mL | 5440-0003 (71-83-01) | 120 mL |
| KPL Detector Block Powder | 5920-0006 (72-01-03) | 10 g | 5920-0006 (72-01-03) | 10 g |
| KPL Phosphatase-labeled Streptavidin (AP-SA) | 5270-0032 (475-3001) | 0.1 mL | 5270-0032 (475-3001) | 0.1 mL |
| KPL 5X Phosphatase Wash Solution | 5910-0022 (50-63-17) | 1000 mL | 5150-0012 (50-63-11) | 200 mL |
| | 5150-0012 (50-63-11) | 200 mL | 5910-0023 (50-63-18) | 100 mL |
| KPL 10X Phosphatase Assay Buffer | 5910-0020 (50-63-12) | 200 mL | 5910-0021 (50-63-13) | 50 mL |
| CDP-Star® Chemiluminescent Substrate | 5430-0033 (50-60-03) | 100 mL | 5430-0034 (50-60-04) | 30 mL |

Sufficient reagents are provided with 5910-0028 (54-31-01) and 5910-0029 (54-31-02) to test approximately 2000 cm$^2$ of membrane (20 each of 10 cm x 10 cm blots) and 500 cm$^2$ of membrane (5 each of 10 cm x 10 cm blots), respectively. Store the KPL 5X Phosphatase Wash Solution at room temperature and the remaining kit components at 2-8°C. Reagents are stable for a minimum of one year when stored as directed.

## Required Supplies and Equipment Not Included

- Biotin-labeled nucleic acid probe
- Herring Sperm DNA
- Ethidium bromide
- 20X SSPE
- SDS
- UV Transilluminator
- UV crosslinker or vacuum oven
- Capillary transfer apparatus
- Waterbath or hybridization oven
- Heat-sealed hybridization bags (Catalog No. 5960-0026) or hybridization bottles
- X-ray film and film cassettes or a chemiluminescent imaging system
- Positively charged nylon or nitrocellulose membrane
- DEPC treated water (For Northern Blotting)
- 37% (v/v) Formaldehyde stock solution (for Northern Blotting)
- 10X MOPS Buffer (See Buffer Preparation for Northern Blotting)
- RNA sample loading buffer (See Buffer Preparation for Northern Blotting)
- Agarose
- Circulating horizontal electrophoresis tank (for Northern Blotting)
- 20X SSC
- NaOH stock solution

**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.**
**⇒ Formaldehyde is a suspected nose, nasopharynx and liver carcinogen. It is toxic both through inhalation and ingestion. Its use should be restricted to a fumehood.**
**⇒ For Northern Blotting: RNA is extremely susceptible to RNases. All tubes and tips should be autoclaved prior to use and all glassware, equipment and trays used should be cleaned using solutions like RNase Away or a 0.2N NaOH solution. Always wear gloves when working with RNA as human skin contains abundant**

## NORTHERN BLOTTING PROTOCOL

Northern blotting refers to the transfer of separated RNA from a gel to a membrane for subsequent detection of the respective RNA transcripts. In the identification of genes and determination of their function, Northern blotting is commonly used to measure the levels of mRNA in tissues. The comparison of these levels to control samples gives insight to relative expression of genes and the impact of various factors on that expression.

While there are a variety of Northern blotting procedures, the protocol described below has been found to give maximum sensitivity and greatest signal-to-noise ratio when using the KPL Detector AP Chemiluminescent Blotting System.

**NOTE ON…Preparation of Biotinylated Probes**

**⇒ Either DNA or RNA probes are suitable for the detection of Northern blots. For the greatest sensitivity, riboprobes are recommended due to the strength of RNA:RNA hybrids relative to DNA:RNA hybrids.**

**⇒ Biotinylated probes may be prepared using the KPL Detector PCR DNA Biotinylation Kit (5910-0031).**

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

**Detector AP Northern Blotting Protocol**
**At A Glance**

**Gel Electrophoresis**
⇓
**Alkaline Transfer**
1 – 3 hour(s)
⇓
**Prehybridization**
1 hour
⇓
**Hybridization**
16 - 18 hours
⇓
**2X SSPE/0.5% SDS washes**
2 x 15 minutes
⇓
**0.2X SSPE/0.5% SDS washes**
2 x 30 minutes
⇓
**1X SSPE wash**
5 minutes
⇓
**KPL Detector Block**
45 minutes
⇓
**KPL AP-Streptavidin**
30 minutes
⇓
**KPL Phosphatase Wash Solution**
3 x 5 minutes
⇓
**KPL Phosphatase Assay Buffer**
2 x 2 minutes
⇓
**CDP-Star**
5 minutes
⇓
**Film Exposure**
2 minutes - 2 hours

## *GEL ELECTROPHORESIS OF RNA*

For optimal electrophoretic resolution and transfer efficiency, a 1 - 1.2% agarose gel is recommended. The following instructions are written as an example of a 1% gel to be cast in a "midi" horizontal gel box (example: Buffer Puffer™, Owl Scientific). Depending on the size of the gel desired, the volume of the components may be adjusted so that the final concentrations are constant.

| STEPS | CRITICAL POINTS |
|---|---|
| 1. For a 1% agarose gel at 100 mL, mix 1 g agarose with 86 mL of DEPC treated water (86% of the final gel volume).<br><br>2. Boil to dissolve the agarose. Cool to 60°C.<br><br>3. In a fume hood, add 10 mL of 10X MOPS buffer to a final concentration of 1X. Swirl to mix.<br><br>4. In a fume hood, add 4 mL of 37% Formaldehyde (12.3M) to a final concentration of 0.45M. Swirl to mix.<br><br>5. In a fume hood, pour the gel into a prepared casting tray to a thickness of 6 mm (approximately 80 mL for the midi apparatus). Insert the comb and allow the agarose to solidify for ~ 45 minutes.<br><br>6. Add RNA sample loading buffer to the RNA at a ratio of 2/1. | *Tare the agarose/water prior to boiling. Afterwards, add back any DEPC treated water that may have boiled off. It is important to maintain accurate agarose concentration.*<br><br>*For accurate time estimation for efficient transfer, it is important to maintain the 6 mm thickness of the gel.*<br><br>*The RNA loading buffer contains formamide to reduce secondary structure in the RNA and ethidium bromide to visualize the RNA on the gel.*<br>*See Buffer Preparation, for RNA loading buffer.* |
| 7. Denature the samples by incubating at 68°C for 10 minutes. Immediately place the samples on ice.<br><br>8. Prepare running buffer: 100 mL 10X MOPS, 40 mL 37% Formaldehyde, 860 mL DEPC Treated Water for a final concentration of 1X MOPS and 0.45M Formaldehyde. Mix well.<br><br>9. Fill the tank and pre-electrophorese the solidified gel at 50 V for 10 minutes.<br><br>10. Turn off the power supply and rinse the wells with running buffer. | *MOPS buffer can concentrate in the wells. They must be rinsed prior to loading of the RNA.* |

| STEPS | CRITICAL POINTS |
|---|---|
| 11. Load the denatured RNA and run the gel at 5 - 7.5 V/cm for approximately 3 hours (dye front should migrate through two-thirds of the gel). | *The RNA needs to run through 2/3 of the gel to get good separation of the rRNA bands to allow analysis of the RNA. The running buffer must be circulated by means of a Buffer Puffer or circulating pump to maintain a constant pH.* |
| 12. Visualize the RNA on an UV Transilluminator. Place a fluorescent ruler next to the gel and photograph it. | *Good quality RNA should appear as two intense band representative of 18s rRNA between 1.8 and 2 Kb and 28s rRNA between 4.6 and 5.2 Kb. These bands should be equal in intensity. If the 18s band is more intense than the 28s band, it may be indicative of degradation.* |

### *ALKALINE TRANSFER OF RNA*

Alkaline transfer is the preferred method for the transfer of RNA onto positively charged nylon membrane. It is more reproducible than high salt overnight transfers. Additionally, it has been shown to be the most efficient method, facilitating transfer within 3 hours regardless of the amount of RNA 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. The nylon membrane should be positively charged.* |
| 2. Treat the gel with 2 gel volumes of alkaline transfer buffer by washing 2 x 10 minutes at room temperature. | *Always use clean forceps to handle membrane.* |
| 3. Assemble a capillary transfer according to standard techniques using the transfer buffer as the solvent. | |
| 4. Transfer time will depend on the amount of RNA loaded on the gel. >3 ug of RNA, transfer 3 hours; 100 ng - 3 ug of RNA, transfer for 2 hours; <100 ng, transfer for 1 hour. | *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.* |
| 5. After transfer, rinse the membrane for 5 min in 5X SSC. Place membrane on filter paper 2 – 4 minutes and fix the RNA to the membrane using a UV cross-linker or vacuum oven according to manufacturer's instructions. | *While most manufacturers claim that nucleic acid transferred to positively charged membrane need not be fixed, KPL has found that sensitivity is greatest after cross-linking or baking.* |
| 6. Store membranes between two pieces of blotting paper and seal in a hybridization bag. Store bag in a cool and dry place. | |

## *PREHYBRIDIZATION & HYBRIDIZATION OF NORTHERN BLOT*

| STEPS | CRITICAL POINTS |
|---|---|
| 1. Place the KPL 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 minimum volume of 0.1 mL/cm$^2$ of membrane is recommended. Use the guidelines listed at the right. | *Use 10 mL of KPL Formamide Hybridization Buffer per 100 cm$^2$ membrane. The volume of buffer may be adjusted depending on the size of the membrane and the vessel used for hybridization.*<br><br>*Hybridization bottles are recommended for hybridization incubations; 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.*<br><br>*For convenience, 10 mL of KPL Hybridization Buffer (regardless of blot size may be used). The hybridization buffer containing probe may be reused and additional KPL 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 100 µg per 1 mL of KPL Formamide Hybridization Buffer that is used. If using SeraCare's KPL Herring sperm DNA, add 5 µL per mL of KPL Formamide 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 RNA 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.*<br><br>*Do not allow membranes to stick together or to the sides of the hybridization bag.* |
| 5. Prehybridize for 1 hour with | |

| STEPS | CRITICAL POINTS |
|---|---|
| constant agitation at the desired hybridization temperature. For DNA probes, hybridization at 42°C is recommended. For RNA probes, hybridization at 65°C is recommended. | |
| 6. Denature probe: DNA probe 95°C for 10 minutes; RNA probe 68°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 (i.e. 500 ng of probe for a 10 mL hybridization). | *Pipette the probe directly into the buffer and not directly on the blot.* |
| 8. Hybridize the membrane with gentle agitation 16 - 18 hours. | |
| 9. Prepare post-hybridization washes:<br><br>2X SSPE/0.5% SDS and 0.2X SSPE/0.5% SDS. Place the 2X solution at room temperature. The 0.2X solution should be equilibrated at least 2 hours at 55°C if washing DNA probes or 65°C if washing RNA probes. | *Washes may be modified to contain different concentrations of SSPE and/or SDS to control stringency of the probe-target hybrid. It is recommended that this be optimized for each individual probe.* |
| 10. Remove the membrane from the KPL Hybridization buffer. Wash 2 X 15 minutes in a generous volume (at least 1 mL per $cm^2$ of membrane) of 2X SSPE/0.5% SDS at room temperature. | *The KPL 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.* |
| 11. Wash with gentle agitation 2 X 30 minutes at the elevated temperature in 0.2X SSPE/0.5% SDS temperature equilibrated wash. | *Completely cover the membrane with the wash solution.* |
| 12. Perform the final wash in 1X SSPE for 5 minutes at room temperature. | *This step is important to eliminate residual SDS carry-over on the membrane to the blocking step which would cause high background.* |
| 13. **Continue immediately with detection.** | *Never allow membrane to dry out during hybridization and detection.* |

***DETECTION OF NORTHERN BLOT***

| STEPS | CRITICAL POINTS |
|---|---|
| 1. Incubate Northern blot with 1X | *See Buffer Preparation for* |

| STEPS | CRITICAL POINTS |
|---|---|
| Detector Block Solution for 45 minutes in a tray approximately the same size as the blot. | *instructions on preparation of 1X KPL Detector Block.*<br><br>*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.* |
| 2. Incubate membrane for 30 minutes in fresh blocking solution with KPL APSA diluted 1/10,000. | |
| 3. Place membrane in a clean container. Wash the membrane in 1X KPL Phosphatase Wash Solution 3 times for 5 minutes each. | |
| 4. Rinse the membrane 2 times for 2 minutes each in 1X KPL Assay Buffer. | *This step is important to remove any detergents from the wash step and to increase the pH of the blot prior to addition of the substrate.* |
| 5. Incubate membrane stationary for 5 minutes in CDP-Star® Chemiluminescent Substrate. Blot membrane on filter paper to remove excess substrate. Place membrane in a hybridization bag or between sheet protectors and expose to X-ray film for an initial exposure of 1 minute. Adjust exposure time for optimal signal-to-noise ratio.<br><br>6. Develop film either manually with a mechanical processor or use a digital imaging system. | *Use 0.05 mL CDP-Star per $cm^2$ of membrane.* |

## 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 and VNTR analysis.

Due to its high sensitivity, the KPL Detector AP Chemiluminescent Blotting Kit is ideal for detection of single copy genes found in target genomic DNA. In fact, these genes may be detected in 5 µg of DNA in as little as 10 minutes. This kit is also suitable for rapid detection of plasmid DNA, PCR products and dot blots.

While there are a variety of Southern blotting procedures, the following protocol is recommended when using the KPL Detector AP 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 PCR DNA Biotinylation Kit.**
**⇒ Quantitate your probe. The concentration of labeled probe should be determined by the quantitation procedure described in SeraCare's Biotinylation Kit. Excessive amounts of probe may result in non-specific signal while the addition of too little probe may result in insufficient signal.**

**KPL Detector™ AP Southern Blotting**
**At A Glance**

**Gel Electrophoresis**
⇓
**Alkaline Transfer**
1 – 3 hour(s)
⇓
**Prehybridization**
1 hour
⇓
**Hybridization**
16 - 18 hours
⇓
**2X SSPE/0.1% SDS washes**
2 x 15 minutes
⇓
**0.2X SSPE/0.1% SDS washes**
2 x 15 minutes
⇓
**2X SSPE wash**
5 minutes
⇓
**Detector Block**
45 minutes
⇓
**AP-Streptavidin**
30 minutes
⇓
**Phosphatase Wash Solution**
3 x 5 minutes
⇓
**Phosphatase Assay Buffer**
2 x 2 minutes
⇓
**CDP-Star**
5 minutes
⇓
**Film Exposure**
2 minutes - 2 hours

| 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.*<br><br>*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.*<br><br>***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.<br><br>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.*<br><br>*Always use clean forceps to handle membrane.* |
| 2. If required, depurinate DNA by incubating the gel in two gel volumes of 0.25N HCl for 10 minutes at room temperature with gentle agitation. | *Depurination is not necessary if the target of interest is <10 Kb. 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/1.5M NaCl for 30 minutes. | *This step is crucial to make the DNA single stranded to allow hybridization of the probe.* |
| 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. | |
| 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. | *Despite claims by other manufacturers, SeraCare recommends fixing of nucleic acids to positively charged membranes by crosslinking or baking to achieve greatest sensitivity.* |
| 8. Store membranes between two pieces of blotting paper and seal in a hybridization bag. Store bag in a cool and dry place. | |

## *PREHYBRIDIZATION & HYBRIDIZATION OF SOUTHERN BLOT*

| STEPS | CRITICAL POINTS |
|---|---|
| 1. Place the KPL 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 minimum volume of 0.1 mL/cm$^2$ of membrane is recommended. Use the guidelines listed at the right. | *Use 10 mL of KPL Formamide Hybridization Buffer per 100 cm$^2$ membrane. The volume of buffer may be adjusted depending on the size of the membrane and the vessel used for hybridization.*<br><br>*Hybridization bottles are recommended for hybridization incubations; 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.*<br><br>*For convenience, 10 mL of KPL Hybridization Buffer regardless of blot size may be used.*<br>*The hybridization buffer containing probe may be reused and additional KPL 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 1 mL of KPL Formamide Hybridization Buffer. If using KPL Herring Sperm DNA, add 10 µL per mL of KPL Formamide 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.*<br>***For best results, seal bag close to the edge of the membrane.***<br>*Do not allow membranes to stick together or to the sides of the hybridization bag.* |
| 5. Prehybridize 1 hour at 42°C with | |

| STEPS | CRITICAL POINTS |
|---|---|
| constant agitation.<br><br>6. Denature the DNA probe at 95°C for 10 minutes. Immediately place on ice.<br><br>7. Add the probe to the Prehybridization Buffer at 50 ng per mL of Buffer (i.e. 500 ng of probe for a 10 mL hybridization).<br><br>8. Incubate the membrane with gentle agitation for 16 - 18 hours at 42°C.<br><br>9. Prepare post-hybridization washes: 2X SSPE/0.1% SDS and 0.2X SSPE/0.1% SDS. Place the 2X solution at room temperature. The 0.2X solution should be equilibrated to 55°C at least 2 hours prior to use.<br><br>10. Remove the membrane from the Hybridization buffer. Wash twice for 15 minutes each in a generous volume (at least 1 mL per $cm^2$ of membrane) of 2X SSPE/0.1% SDS at room temperature.<br><br>11. Wash with gentle agitation twice for 15 minutes each at the elevated temperature in the temperature equilibrated 0.2X SSPE/0.1% SDS wash.<br><br>12. Perform the final wash in 2X SSPE for 5 minutes at room temperature.<br><br>13. **Continue immediately with detection.** | *Do not denature probes by alkaline treatment.*<br><br>*Pipette the probe directly into the buffer and swirl. Do not pipette directly onto the blot.*<br><br>*The desired hybridization temperature when using DNA probes is 42°C.*<br><br>*Washes may be modified to contain different concentrations of SSPE and/or SDS to control stringency of the probe-target hybrid. It is recommended that this be optimized for each individual probe.*<br><br>*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.*<br><br>*Generously cover the membrane with the wash solution.*<br><br>*This step is important to eliminate residual SDS carry-over on the membrane to the blocking step, which would cause high background.*<br><br>*Never allow membrane to dry out during hybridization and detection.* |

## *DETECTION OF SOUTHERN BLOT*

| STEPS | CRITICAL POINTS |
|---|---|
| 1. Prepare enough 1X KPL Detector Block Solution for the blocking step and for the KPL AP-SA conjugate dilution. | *Use at least 0.25 mL 1X KPL Detector Block Solution per $cm^2$ membrane. See Buffer Preparation for instructions on preparation of 1X KPL Detector Block.* |
| 2. Incubate Southern blot with 1X KPL Detector Block Solution for 45 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.*<br><br>*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 KPL AP-SA conjugate at 1/10,000 in fresh 1X KPL Detector Block. Mix well. | *Example: For 25 mL of blocking solution, add 2.5 µL conjugate.* |
| 4. Pour off the blocking/diluent solution (from step 2) from the membrane and add the diluted KPL AP-SA solution. Incubate for 30 minutes. | |
| 5. Place membrane in a clean container. Wash the membrane 3 times in 1X KPL Phosphatase Wash Solution for 5 minutes each. | *See Buffer Preparation for instructions on preparation of 1X KPL Phosphatase Wash Solution.* |
| 6. Rinse the membrane 2 times for 2 minutes each in 1X KPL Phosphatase Assay Buffer. | *It is important to remove any detergents from the wash step and to increase the pH of the blot prior to addition of the substrate.*<br><br>*See Buffer Preparation for instructions on preparation of 1X KPL Phosphatase Assay Buffer.* |
| 7. Incubate membrane stationary for 5 minutes in CDP-Star® Chemiluminescent Substrate. Blot membrane on filter paper to remove excess substrate. Place membrane in a hybridization bag or between sheet protectors and expose to X-ray film for an initial exposure of 1 minute. Adjust exposure time for optimal signal-to-noise ratio. | *Use 0.05 mL CDP-Star per $cm^2$ of membrane.* |
| 8. Develop film either manually with a mechanical processor or use a digital imaging system. | |

## STRIPPING AND RE-PROBING BLOTS

Membranes hybridized with biotinylated probes can be stripped and re-probed after detection with CDP-Star 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.

### *NORTHERN BLOTS*

**STRIPPING A DNA PROBE**

1. Pour a boiling 0.1% SDS solution onto the membrane and shake for 5 minutes.
2. Discard the solution and immediately add fresh boiling 0.1% SDS solution to the membrane. Continue shaking until the solution comes to room temperature.
3. Rinse the membrane in 1X SSPE for 5 minutes.
4. Reprobe immediately or store the membrane in 1X SSPE at 4°C until ready to use.

**STRIPPING A RNA PROBE**

1 Incubate the membrane in the following solution for 1 hour at 65°C:
- 55 mL of Formamide (37% solution)
- 10 mL of 20X SSPE
- 5 mL of 20% SDS
- 30 mL of DEPC treated water

2 Rinse the membrane in 1X SSPE for 5 minutes.

3 **Reprobe immediately or store the membrane in 1X SSPE at 4°C until ready to use.**

### *SOUTHERN BLOTS*

**STRIPPING A DNA PROBE**

1 Wash membrane in 1X KPL Phosphatase Wash Solution for 5 minutes at room temperature.

2 Incubate membrane in 0.2N 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.**

**CDP-Star® Chemiluminescent Substrate**

⇒ CDP-Star can be used with nylon and PVDF membranes. Use on nitrocellulose requires an additional component.

⇒ CDP-Star can be used with Chemiluminescent Imagers.

⇒ CDP-Star reaches peak light emission at 2 - 4 hours persisting for several days, allowing the user multiple film exposures. For most applications, exposures of one hour or less provide sufficient sensitivity.

⇒ The exposure time to film will enable optimization of the signal-to-noise ratio. **Overexposure will increase background and eventually result in a black blot.**

⇒ If there is background after the initial 5 - 10 minute film exposure, re-expose the blot to film for 1 minute.

⇒ If the signal-to-noise ratio is low after the initial 5 - 10 minute film exposure, leave the blot in the film cassette without film for 1 - 3 hours, then re-expose to film for 1 - 10 minutes. Allowing CDP-Star to reach its maximum light emission before exposure to film may enhance signal relative to noise.

⇒ Do not allow CDP-Star to contact film. This will cause dark spots to appear on the film.

## TROUBLESHOOTING GUIDE FOR AP 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 KPL 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 1/10,000, *i.e.* 5 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 and 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 intact; 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).<br><br>Make sure to include sheared, denatured Herring sperm DNA in the hybridization cocktail. |
| • Non-specific hybridization<br><br>• 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 DNA probe. |
| • 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 0.5N NaOH/1.5M 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 |

| Possible Cause | Corrective Measure |
|---|---|
| | 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. |
| • Too much KPL Detector Block powder was used | Add the powder 0.2% w/v. Too much powder up to 1% will decrease sensitivity. |

**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 KPL Detector™ AP Chemiluminescent Blotting Kit when volumes are used as indicated. If desired, increased working volumes may be used; however, additional reagents will be necessary. Individual components may also be purchased separately. For optimal results, recommended preparation of kit buffers are detailed below.

**1X KPL Detector™ Block Solution – to be prepared fresh daily**

| STEPS | CRITICAL POINTS |
|---|---|
| 1. Based on the total desired 1X KPL Detector Block volume, weigh out 0.2% w/v KPL Detector Block Powder for detection with AP and CDPStar. | *If the block solution is not prepared daily, sensitivity could be reduced and background will increase.* |
| 2. Place the KPL 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 KPL Detector Block volume. Shake the container vigorously until the powder is fully solubilized. | *Conical tubes are not recommended in the preparation of 1X KPL Detector Block. If used, the solution may be vortexed to remove any packed KPL Detector Block Powder from the bottom of the tube.* |
| 3. Dilute the solution with 5X KPL Detector Block Solution 1/5 v/v. Ex., for 50 mL of 1X KPL Detector Block:<br>KPL Detector Block Powder - 0.1 g<br>Molecular Biology Grade $H_20$ - 40 mL<br>5X KPL Detector Block Solution - 10 mL | *Insure that all KPL Detector Block Powder is in solution to avoid speckling patterns on the blot or insufficient blocking that may occur as a result of unsolubilized powder. The amount of powder used can be increased to decrease background. However, too much powder will reduce sensitivity.* |

**KPL Phosphatase Wash Solution**

| STEPS | CRITICAL POINTS |
|---|---|
| 1. Dilute 1 part 5X KPL Phosphatase Wash with 4 parts molecular biology grade water. Mix well. | *SDS may fall out of solution in the 5X concentrate. Simply place the bottle in a 37 – 65°C water bath for a few minutes and mix until the solution is homogenous.* |

**KPL Phosphatase Assay Buffer**

| STEPS |
|---|
| 1. Dilute 1 part 10X KPL Assay Buffer with 9 parts molecular biology grade water. Mix well. |

**10X MOPS Buffer**

| STEPS |
|---|
| 1. Mix together the following:<br><br>0.2M MOPS, pH 7.0<br>0.05M Sodium Acetate<br>10mM EDTA, pH 8.0<br>1 Liter of DEPC Treated Water<br><br>2. Sterile filter solution. |

**RNA Sample Loading Buffer**

| STEPS |
| --- |
| 1. Mix together the following:<br><br>62.5% Deionized Formamide<br>1.14M Formaldehyde<br>200 ug/mL Bromophenol Blue<br>200 ug/mL Xylene Cyanole<br>1.25X MOPS Buffer<br>50 ug/mL Ethidium Bromide |

**20X SSC**

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

**20X SSPE**

| STEPS |
| --- |
| 1. Mix together the following:<br>3.0M NaCl<br>200mM $NaH_2PO_4$<br>20mM EDTA, pH 7.4<br><br>2. Sterile filter or autoclave solution<br>50 ug/mL Ethidium Bromide |

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## RELATED PRODUCTS

| Product | Size | Cat. No. |
|---|---|---|
| KPL Detector™ PCR DNA Biotinylation Kit | 30 reactions | 5910-0031 (60-01-01) |
| Detector HRP Chemiluminescent Blotting Kit | 20 blots | 5910-0027 (54-30-00) |
| KPL 20X SSC | 1 Liter | 5960-0021 (50-86-05) |
| KPL Herring Sperm DNA, sheared & denatured | 40 mg | 5920-0003 (60-00-14) |
| KPL Biodyne®B Membrane | 20 cm x 1 m roll | 5960-0025 (60-00-50) |
| KPL Hybridization Bags | 50 bags | 5960-0026 (60-00-51) |
| KPL Phosphatase-Labeled Streptavidin | 1.0 mL | 5950-0005 (475-3000) |
| KPL 5X Detector Block | 20 mL | 5920-0004 (71-83-00) |
| KPL Formamide Hybridization Buffer | 2 x 120 mL | 5960-0023 (50-86-10) |
| KPL 5X Phosphatase Wash Solution | 600 mL | 5960-0018 (50-63-15) |
| KPL 10X Phosphatase Assay Buffer | 200 mL | 5960-0017 (50-63-14) |
| KPL GeneRuler™ Biotinylated DNA Ladder | 100 µL | 5960-0029 (600-0008) |

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