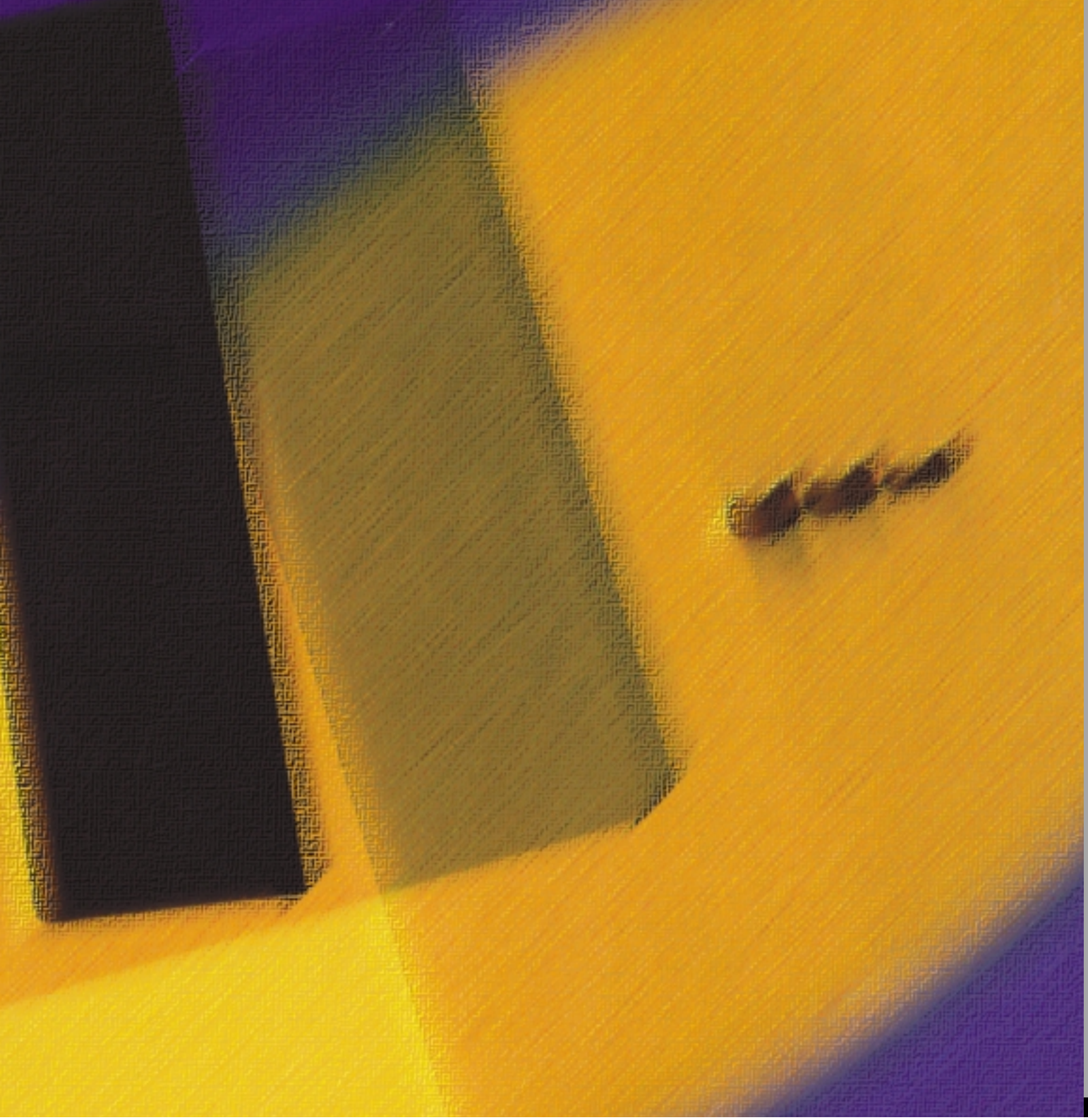

DETECTOR™ SYSTEMS

Technical Guide for Non-Radioactive Nucleic Acid Labeling and Detection

KPL

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Preface

Detector™ is a comprehensive line of kits and reagents for non-radioactive labeling and detection of nucleic acids. These systems have been developed to eliminate the need for radioisotopes without compromise to the high sensitivity associated with their use. Additionally, Detector products address the background issues that have been typical of past chemiluminescent methods through the use of unique hybridization and blocking solutions and well-defined assay conditions. The result is an optimized approach to nucleic acid blotting applications that is fast, efficient and reliable, producing publication quality blots with superior signal:noise ratio. Included in the Detector product line are kits for:

- Biotin labeling of DNA and RNA probes
  - Detector Random Primer DNA Biotinylation Kit
  - Detector PCR DNA Biotinylation Kit
  - Detector RNA *in vitro* Transcription Biotinylation Kit
- Southern Blotting
  - Detector AP Chemiluminescent Blotting Kit
  - Detector HRP Chemiluminescent Blotting Kit
- Northern Blotting
  - Detector AP Chemiluminescent Blotting Kit
- *In situ* Hybridization
  - DNADetector™ Chromogenic *in situ* Hybridization Kit
  - DNADetector Fluorescent *in situ* Hybridization Kit

This *Technical Guide to Non-Radioactive Nucleic Acid Labeling and Detection* is designed as a primer for those laboratories evaluating chemiluminescent detection for the first time; it serves as a resource for comparing techniques, selecting the appropriate products and conducting experiments. In addition to an overview of the applications, complete protocols are included along with special notes and recommendations to ensure maximum performance. For those with more extensive experience, the guide acts as a quick reference and troubleshooting tool.

Non-rad vs. ^{32}P

A variety of methods have been developed to detect specific nucleic acid sequences immobilized on membranes (i.e., dot/slot blot, Southern blot, Northern blot, South-Western blot, colony and plaque lifts) and localized *in situ* in cells and tissues. ^{32}P has traditionally been used due to the intensity of signal it produces and, thus its ability to facilitate the detection of small amounts of biomolecules on blots. However, ^{32}P is not without its shortcomings. These include issues associated with handling and disposing of hazardous material, long exposure times and short half-life, limiting the stability of probes.

In recent years, non-radioactive nucleic acid labeling and detection methodologies have become available in response to a desire by researchers and their institutions to move away from the use of radioisotopes. Advancements made in the areas of chemiluminescence and fluorescence have allowed for an easier transition. In non-radioactive assays, signal is generated through an enzymatic reaction with a chemiluminescent or chromogenic substrate; alternatively, detection can occur through the appropriate excitation and emission of a fluorophore-labeled probe. For those laboratories seeking replacement technology to ^{32}P without significant investment in instrumentation, chemiluminescent detection enables equivalent results, easily and quickly captured on digital imaging systems or X-ray film shortly after exposure. It is now possible to detect femtogram quantities of nucleic acid in as little as 10 minutes when a hapten and reporter molecule are used in conjunction with a chemiluminescent substrate. The hazards and regulatory issues surrounding ^{32}P-based detection are no longer a trade-off for sensitive, reproducible results.

Optimal non-isotopic nucleic acid detection depends primarily on three variables: 1) the molecule or compound used to label the probe, 2) hybridization conditions, and 3) the detection method.

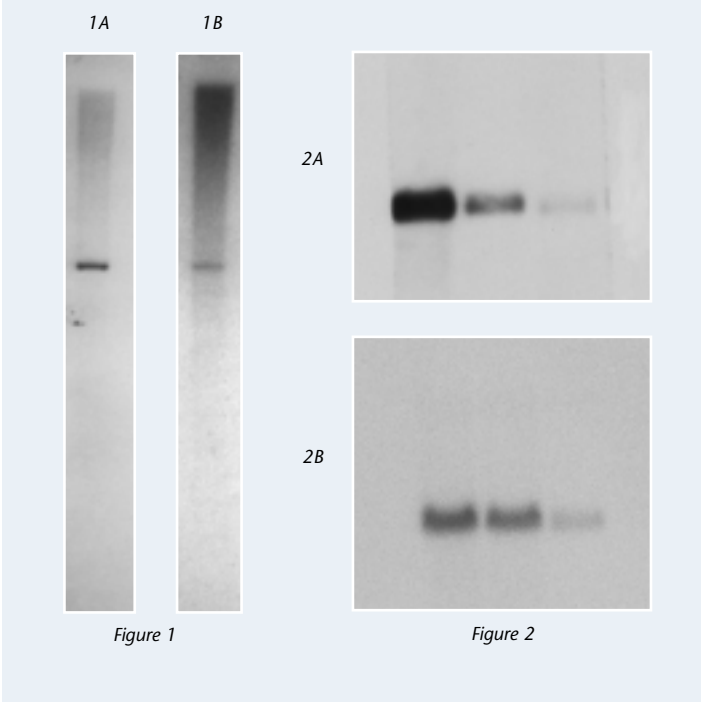
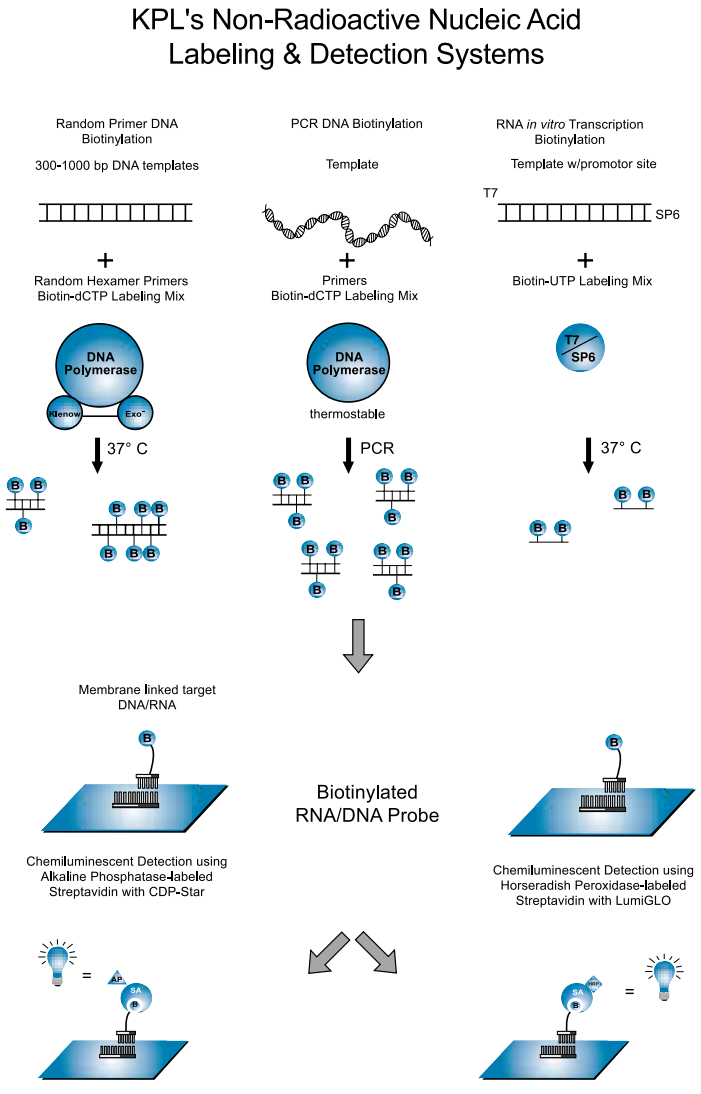


Figure 1 Figure 2

Figure 1: Comparison of Southern Blot Detection using Detector Labeling and Detection vs. ^{32}P. Detection of single copy gene, n-myc, from 5 µg of human genomic DNA. Panel 1A: Blot detected with a biotinylated n-myc probe and Detector AP Chemiluminescent Blotting Kit in a 10 minute film exposure. Panel 1B: Detection of the same gene with ^{32}P-labeled probe, 16 hour exposure.

Figure 2: Comparison of Southern Blot Detection using Detector Labeling and Detection vs. ^{32}P. HeLa cell total RNA detected using an in vitro transcribed biotinylated ß-actin RNA probe and the Detector AP Chemiluminescent Blotting Kit in a 10 minute film exposure (2A) and a ^{32}P-labeled probe in a 3.5 hour film exposure (2B).



Probe Labeling

Non-radioactive labeling can be accomplished by direct or indirect labeling methods. The former includes direct incorporation of fluorescent tags, or cross-linking enzyme molecules directly to nucleic acid. Indirect labeling involves the incorporation of nucleotides tagged with a hapten such as biotin or digoxigenin (DIG) during synthesis of the probe. Used in standard blotting and hybridization procedures, labeled probes that hybridize to a target sequence are detected with streptavidin (biotin) or anti-DIG monoclonal antibody (DIG) conjugated to an enzyme, usually phosphatase or peroxidase. Enzyme activity can be detected either by a chemiluminescent reaction whereby results are captured on X-ray film, or through formation of a color precipitate deposited directly on the membrane. Nucleic acid probes generated by these means are stable for at least one year, in contrast to the short half-life of ^{32}P-labeled probes. Additionally, they may be handled and disposed of without the concerns of hazardous radioactivity.

Biotin is commonly used because it binds to avidin or streptavidin with high affinity (Kd=10^{-15} M), the strongest of any non-covalent bond.[1] This affinity constant is significantly higher than that between DIG and an anti-DIG monoclonal antibody, and contributes to the higher reproducibility of the biotin/streptavidin system. Biotin can be incorporated into nucleic acid probes in the form of a biotinylated nucleotide by enzymatic methods. *(Details of each labeling method described in Chapter 2)* Biotin may also be attached by direct means using intercalation or photo-activatable groups. Biotinylation rarely interferes with biological activity, and linker arms between the biotin and the probe minimize steric interference. The high affinity association between biotin and streptavidin enhances sensitivity because greater wash steps can be carried out reducing subsequent background problems when compared to DIG (See Figure 3 and Figure 4).

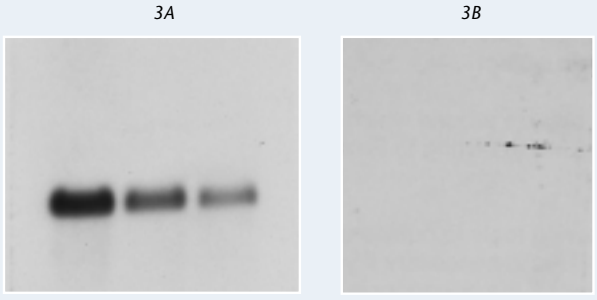


Figure 3

Figure 3: Comparison of Detector Labeling and Detection vs. Digoxigenin Systems for Northern Blots. HeLa cell total RNA loaded at 10, 5 and 1 µg, transferred to Biodyne® B Nylon Membrane via alkaline transfer and detected with alternative non-radioactive methods. 3A: 838 bp ß-actin DNA probe biotinylated by Detector Random Primer DNA Biotinylation Kit and detection with Detector AP Chemiluminescent Blotting Kit with CDP-Star® in a 2 minute film exposure. 3B: Same probe DIG-labeled with DIG High Prime Labeling Kit and detected using DIG Luminescent Detection Kit with CSPD Substrate in 10 minute exposure. Extended exposure time produced signal at 36 hours with high background. (Data not shown).

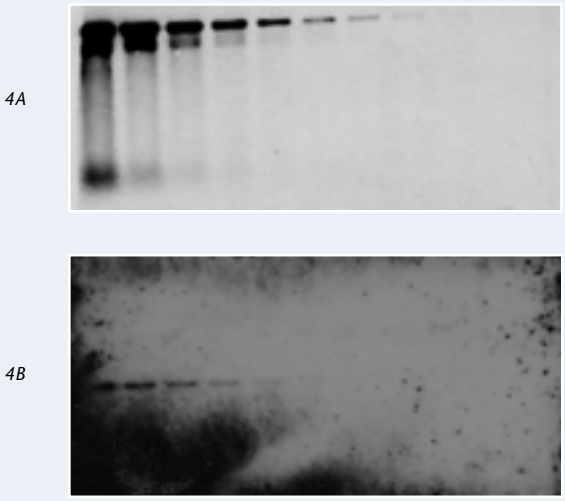


Figure 4

Figure 4: Comparison of Detector Labeling and Detection vs. Digoxigenin System for Southern Blots. Two-fold serial dilutions of Lambda-HindIII beginning at 25 ng were electrophoresed and transferred by alkaline transfer to positively charged nylon membrane. 500 bp DNA probes were labeled with biotin using Detector PCR DNA Biotinylation Kit or DIG using DIG High Prime DNA Labeling Kit. 4A: Detection of biotin probe with Detector AP Chemiluminescent Blotting Kit in a 10 minute film exposure. 4B: Detection of DIG probe with DIG Luminescent Detection Kit after a 10 minute exposure.

KPL's line of Detector Kits described in this guide is based on the biotin/streptavidin system. Because of the high efficiency the Detector Labeling Kits deliver, the biotinylated probes produced through random priming, PCR and *in vitro* transcription can be used to detect single copy genes, low expressed mRNA, positive clones in bacterial colonies or plaques as well as localized DNA in tissues and cells.

Hybridization Conditions

Once the probe is labeled and quantitated with an appropriate hapten, it can be hybridized to the target nucleic acid through complementary base pairing. The two strands of a DNA double helix are held together by relatively weak hydrogen bonds that can be broken or denatured by heating or subjecting them to extremes of pH. When incubated under the appropriate conditions, the complementary strands will re-associate or re-nature to form a double stranded structure that results from restoration of the complementary hydrogen bonds. This process is called hybridization and refers to the formation of sequence-specific, base-paired double helices. Hybridization will occur between any two single-stranded nucleic acid chains (DNA-DNA, DNA-RNA, RNA-RNA) provided that they have complementary nucleotide sequences.

Table 1: Hybridization Solution Components and Effects

Component	Effect	Action
Sodium ion concentration	Favors formation of hydrogen bonds	Increasing [Na^+] increases Tm.
Detergent (SDS, Sarkosyl, Tween)	Prevents nonspecific ionic interactions of probe with the membrane.	Insufficient detergent may result in background. Excessive detergent may reduce sensitivity.
Nonspecific nucleic acid (herring or salmon sperm DNA)	Blocks nonspecific hybridization of nucleic acid probe.	Addition of nonspecific nucleic acid can decrease nonspecific background by binding to non-specific regions on the membrane. Also, if the probe is a whole genomic probe, the herring sperm will block repetitive elements. Excessive amounts of nonspecific nucleic acid will reduce sensitivity.
Formamide (deionized)	Lowers the Tm of the nucleic acid hybridization.	Formamide concentration up to 50% decreases the Tm of the nucleic acid hybridization and reduces the optimum hybridization temperatures.
Protein solution (Blotto, Denhardt's)	Blocks nonspecific binding of probe to the membrane.	May reduce or increase background depending on the membrane used.
Polymer accelerant (PEG, Dextran sulfate, PVP)	Increases probe concentration by lowering the active water content.	May reduce or increase background depending on the membrane used.

Tm = melting temperature

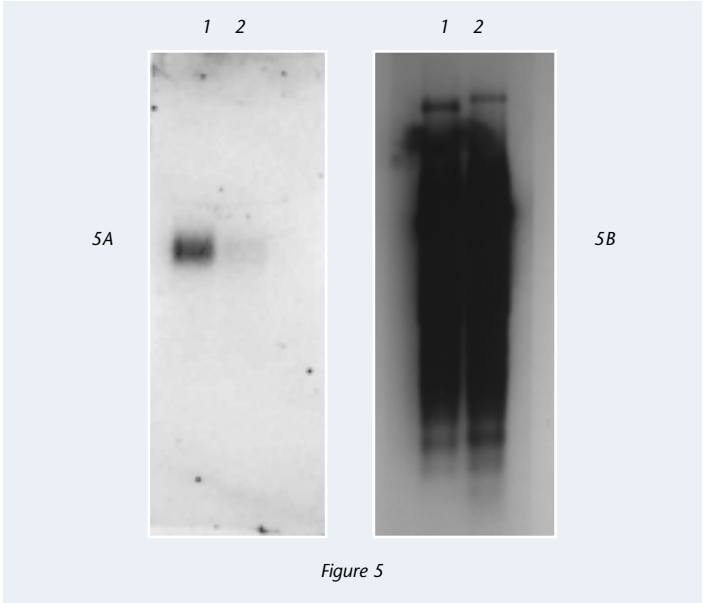


Figure 5

Figure 5: Detection of a Low Expressed Transcript with Varying Hybridization Conditions. Duplicate lanes of 5 µg of total RNA from WEHI-231 untreated and anti-IgM treated cells were electrophoresed on a 1% formaldehyde gel and transferred by a 2 hour alkaline method to Biodyne B Nylon Membrane. The membrane was cut in half and hybridized with a biotinylated c-myc riboprobe in either formamide or aqueous hybridization buffer. Detection was carried out using the Detector AP Chemiluminescent Blotting Kit. 5A: When using a formamide-based solution, the c-myc gene was observed as a single band in the control sample (1) and the down-regulated treated sample (2). 5B: Significant non-specific binding of the probe to the total RNA resulted on the blot hybridized in an aqueous hybridization solution.

The stability of the nucleic acid duplexes and the stringency of hybridization conditions determine the efficiency of hybridization. Several factors destabilize these hybrids by lowering their melting temperature. These factors can be adjusted to favor formation of specific hybrids with minimal interference from less specific hybrids. In any assay system, increasing stringency improves specificity with a corresponding loss in sensitivity; conditions should be optimized for specific applications. Table 1 describes typical hybridization solution components and their effects on hybridization efficiency.

The hybridization solution contained in Detector kits was formulated to include formamide. As a destabilizer formamide lowers the melting temperature of hybrids, increasing the stringency of the probe to target binding. Use of this agent results in minimal nonspecific hybridization; less optimization of washes is required by the end user. Unlike aqueous hybridization solutions, buffers containing formamide effectively minimize background to allow subsequent detection of single copy genes and low expressed transcripts. (Figure 5) Thus, these types of solutions can be applied more universally. Blotting procedures may also be expedited through the use of Formamide Hybridization Buffer, reducing an overnight hybridization to 2 hours without impact on signal:noise ratio. Note that this is acceptable when detecting plasmid DNA or moderately expressed transcripts; however, overnight incubations are required for greatest sensitivity when detecting low copy genomic DNA and rare mRNA.

Detection Methods

Detection can be mediated either directly when using fluorescent haptens or indirectly with the use of binding proteins like antibodies or avidin/streptavidin as in the Detector system. The specific antibody or binding protein is coupled to an enzyme or fluorochrome and subsequently visualized

Table 2: Non-Radioactive Detection Systems for Membrane Hybridization

Enzyme	Substrate	Product/Detection Method
Horseradish Peroxidase	TMB	Blue color / visual
	4CN	Purple color / visual
	DAB	Brown color / visual
	LumiGLO®	Light emission/ X-ray film; Digital chemi–imaging systems
Alkaline Phosphatase	BCIP/NBT	Purple color / visual
	CDP-STAR®	Light emission / digital imaging systems, X-ray film

Table 3: Non-Radioactive Detection Systems for *in situ* Hybridization

Enzyme	Substrate/Fluorochrome	Product/Detection Method
Horseradish Peroxidase	DAB	Brown color / light microscopy
	AEC	Red color / light microscopy
	TrueBlue™	Blue color / light microscopy
Alkaline Phosphatase	BCIP/NBT	Purple color / light microscopy
	Fast Red	Red color / light microscopy
Fluorochromes	FITC	Green fluorescence / fluorescence microscopy
	TRITC; CY™3; Texas Red®	Red fluorescence / fluorescence microscopy

through a variety of signal-generating systems. In enzymatic detection, the enzyme reacts with a specific substrate to produce either a colored or luminescent product. Tables 2 and 3 provide more detailed information on alternative enzyme/substrate methods for membrane and *in situ* hybridization.

Given the advantages outlined in the earlier discussion, Detector is fundamentally based on a chemiluminescent detection schema for the visualization of nucleic acids in blotting applications. Two substrate systems are available for use with alkaline phosphatase (AP) and horseradish peroxidase (HRP) enzymes; selection is dependent on the performance requirements of the assay.

CDP-Star, a chloro-substituted 1,2 dioxetane AP chemiluminescent substrate, offers the highest sensitivity for detecting even the smallest amounts of nucleic acids. Single copy genes in genomic DNA and low expressed messages in as little as 1µg of total RNA are visualized on film in no more than a 10-minute exposure. Alkaline phosphatase catalyzes the removal of the phosphate from CDP-Star to yield a moderately stable intermediate, which then spontaneously decays and emits light at 461 nm. The chemiluminescent signal persists for days on nylon membranes, permitting multiple film exposures.

Sensitive detection may also be achieved with LumiGLO, Chemiluminescent Peroxidase Substrate. A luminol-based substrate, LumiGLO is converted to an excited intermediate dianion by HRP in the presence of hydrogen peroxide. The dianion emits light on return to its ground state, detecting positive reaction sites in minutes; signal continues for 1-2 hours. It serves as an economical alternative to CDP-Star, suitable for plasmid or genomic blots and ideal for bacterial colony and plaque hybridization where CDP-Star is not recommended. While production of signal is not at the accelerated rate of CDP-Star, detection of 0.3 pg DNA can readily be achieved after just 15 minutes on film.

Although a superior non-radioactive method for membrane applications, chemiluminescence is not practical for the detection of DNA *in situ*. Rather, a better approach to visualizing DNA in cells, tissues and metaphase chromosomes relies on chromogenic and/or fluorescent systems. KPL's TrueBlue substrate was developed to react with HRP-streptavidin conjugates, producing a very fine precipitate versus the large clumps of color often yielded from BCIP/NBT. As a result, superior resolution of localized DNA is obtained. The brilliant blue stain gives excellent contrast to red counterstains used to provide nuclear detail. Sensitivity is equivalent to fluorescent *in situ* hybridization and results are permanent.

Fluorescent *in situ* hybridization (FISH) has grown in prevalence in the past decade, particularly with the availability of new and more sensitive fluorescent tags. For instance, cyanine dyes produce bright, intense colors 5-10 times more fluorescent than fluorescein-labeled probes[2]. CY-labeled streptavidin conjugates can be used with biotin probes to detect DNA in cells. KPL's DNADetector™ Fluorescent *in situ* Hybridization Kit contains CY3-Streptavidin; alternatively other cyanine-streptavidin conjugates may be used. These probes are more photostable than FITC or TRITC with bright fluorescence observed 6 months after staining. Using the same filters as TRITC, CY3 excites at 552 nm and emits orange color at 570 nm.

The remainder of *The Technical Guide to Non-Radioactive Labeling and Detection of Nucleic Acids* consists of the detailed procedures for performing specific applications employing biotin and Detector kits. The following table (Table 4) summarizes the properties of the Detector product line, assisting in the selection of the appropriate system for your needs.

Table 4: Choosing KPL Detector Kits

Detector Labeling Kits	Catalog Number	Kit Size	Labeling Method	Sensitivity	Applications
Random Primer DNA Biotinylation Kit	60-01-00	30 reactions	Exo- fragment of Klenow DNA polymerase extends primers by catalyzing the addition of nucleotide triphosphate to the nascent probe from a mixture that includes biotin-dCTP	Only 100 ng purified template needed per reaction.	Southern, Northern and dot blotting; colony and plaque hybridization and *in situ* hybridization.
PCR DNA Biotinylation Kit	60-01-01	30 reactions	Incorporation of biotin-dCTP via a thermostable DNA polymerase in the polymerase chain reaction.	As little as 1 ng genomic template DNA can be amplified and labeled.	Southern, Northern, dot blotting; colony and plaque hybridization, and *in situ* hybridization.
RNA *in vitro* Transcription Biotinylation Kit	60-01-02	20 reactions	DNA located downstream of the RNA polymerase promoter site is copied in a strand – specific manner into a RNA transcript in the presence of ribonucleotides (biotin-UTP) and either T7 or SP6 RNA polymerase.	One reaction generates enough probe to hybridize 48-96 blots.	Southern and Northern blotting; mRNA *in situ* hybridization.
Detector Detection Kits	**Catalog Number**	**Kit Size**	**Detection Method**	**Sensitivity**	**Applications**
AP Chemiluminescent Blotting Kit	54-30-01 54-30-02	2000 cm^2 500 cm^2	AP-SA and CDP-Star Chemiluminescent Substrate	Detection of single copy genes in 5 µg of genomic DNA, low expressed message in 1-5 µg total RNA or ß-actin in just 50 ng of total RNA after a 10-minute film exposure.	Northern Blotting Genomic Southern blotting of single copy genes, plasmid DNA, dot blots, and PCR products.
HRP Chemiluminescent Blotting Kit	54-30-00	2000 cm^2	HRP-SA and LumiGLO Chemiluminescent Substrate	Detection of 0.3 pg DNA with a 15-minute film exposure.	Southern blotting, bacterial colony and plaque hybridization dot blots.
Chromogenic *In situ* Hybridization Kit	60-03-00	50 samples	HRP-SA and TrueBlue peroxidase substrate: Orcein and Eosin Y counterstains	Sensitivity equivalent to FISH.	DNA detection in cells, tissues, and metaphase chromosomes.
Fluorescent *In situ* CY3/DAPI Hybridization Kit	60-05-00	50 samples	CY3-SA: DAPI counterstain	5-10 times greater fluorescence than FITC/TRITC labeled probes using the same filters.	DNA detection in cells, tissues, and metaphase chromosomes.

References

[1] Brzofsky, JA (1991) *"Antigen and antibody interactions and monoclonal antibodies,"* Fundamentals of Immunology, 3rd Edition, WE Paul.

[2] Yurov, Y.B. *et.al. Human Genetics,* 97, 390-398 (1996).

Introduction to KPL's Detector™ Labeling Kits

KPL offers three labeling approaches to the generation of biotinylated nucleic acid probes:

- Detector Random Primer DNA Biotinylation Kit
- Detector PCR DNA Biotinylation Kit
- Detector RNA *in vitro* Transcription Biotinylation Kit

Both random primer and PCR-mediated biotin labeling results in the net synthesis of DNA and amplification. Random primed labeling is catalyzed by Klenow polymerase, the large fragment of *E. coli* DNA polymerase. The Klenow polymerase lacks 5'→3' exonuclease activity of the holoenzyme but still contains the 5'→3' polymerase as well as the 3'→5' exonuclease proof-reading activity. During the polymerization reaction, Klenow polymerase incorporates not only the non-modified deoxynucleotides but also the hapten-modified substrates (e.g., biotin-dCTP), resulting in a DNA probe with high specific activity. PCR-mediated labeling of probes with biotin allows simultaneous amplification and labeling of DNA. Thermostable *Taq* DNA polymerase drives the PCR reaction, incorporating biotin into the PCR product via modified deoxynucleosite triphosphates. The end product is homogeneously labeled hybridization probes that can detect sub-picogram amounts of target sequences on blots.

Probes generated by either random priming or PCR are typically used in Southern blots; they are also suitable for Northern blots. While DNA probes are commonly used in the detection of nucleic acids on membranes, RNA probes are an advantageous alternative and should be considered particularly when the visualization of low expressed genes is desired. In these cases, detection with riboprobes can be approximately 10 times more sensitive than DNA probes. This increase is accounted for by the great affinity of a riboprobe for the complementary sense strand of the mRNA being detected and the resulting higher stability of the RNA:RNA bond after hybridization.[1] Additionally, single stranded RNA probes are not subject to the self-annealing that double-stranded DNA probes are, which decreases the availability of the DNA probes to bind to the immobilized target.

Single-stranded RNA probes can be generated by *in vitro* transcription from RNA polymerase promoters such as SP6, T7 or T3. DNA located downstream of the RNA polymerase promoter site is copied in a strand-specific manner into an RNA transcript in the presence of ribonucleotides and the appropriate RNA polymerase. Because of the nature of transcription reactions, many copies of RNA are produced from the template DNA in a short time. Transcripts can be labeled during synthesis by incorporation of biotin during transcription. The incorporation of biotin-UTP by SP6 or T7 polymerase is very efficient, resulting in highly labeled RNA probes.

The following protocols describe in detail the process of biotin labeling nucleic acid probes by random priming, PCR labeling and *in vitro* transcription using KPL's Detector Biotin Labeling Kits. For additional assistance while using these systems, the labeling section of the Troubleshooting Chapter (Chapter 6) may be referenced.

Detector Random Primer DNA Biotinylation Kit (Cat. No. 60-01-00)

The Detector Random Primer DNA Biotinylation Kit provides a method for biotinylating DNA probes through incorporation of biotin-dCTP during random-primer extension.[2, 3] Six base random sequence oligonucleotides serve as primers for replication of the template DNA. The Exo- fragment of Klenow DNA polymerase extends the primers by catalyzing the addition of nucleotide triphosphate, from a mixture that includes biotin-dCTP, to the nascent probe. Large quantities of biotinylated DNA probes can be generated from a small quantity of template DNA. This labeling method results in the net synthesis of DNA. The use of Exo- Klenow polymerase allows for longer labeling reactions without the risk of degradation of the oligonucleotides.

The components of this kit are optimized to maximize amplification of template DNA and sensitivity of target detection.[4]

Template requirements – Optimal labeling occurs on templates that range from 300-1000 bp. The probe fragments generated vary in length from 100 to 1,000 bases, with the average length being approximately 300 base pairs.

Probe storage – Stable for at least one year when stored at -20°C. Because biotinylated probes stick to normal microcentrifuge tubes, it is recommended that probes be stored in low retention or siliconized tubes.

Probe quantitation – A pre-labeled quantitation standard is included for determining the relative amount of DNA synthesized during the labeling reaction. The concentration of probe to be used in the hybridization buffer must be optimized for greatest sensitivity and minimal background.

- Serial dilutions of quantitation standard and newly labeled probe are fixed to positively charged nylon membrane.
- The standard and probe are detected using enzyme-labeled streptavidin, and chemiluminescent or chromogenic substrate.
- Endpoint sensitivity of the samples is compared to the standard to determine the amount of probe generated.

Materials and Equipment

Kit Components	Product Code	Volume
2.5X Random Primer Solution	600-0001	700 µL
10X dNTP Mixture	600-0002	175 µL
Klenow DNA Polymerase (Exo-fragment)	600-0003	35 µL
Control Template DNA	600-0004	25 µL
Stop Buffer	600-0005	250 µL
DEPC Treated Water	50-86-03	1.0 mL
Quantitation Standard	600-0007	150 µL

Sufficient reagents are provided to perform 30 labeling reactions when following the protocol described below. Kit components are stable for a minimum of 1 year. Reagents must be stored at –20°C and kept on ice during use. Do not store kits in a frost-free freezer.

Required Supplies and Equipment Not Included

- 37ºC water bath or heat block
- 1X TE (See Appendix for preparation)
- 20X SSC (Catalog No. 50-86-05 or see Appendix for preparation)
- Low retention microcentrifuge tubes
- Microcentrifuge
- UV crosslinker or vacuum oven
- Positively charged nylon (Biodyne B, Catalog No. 60-00-50) or nitrocellulose membrane
- For Probe Quantitation - Detector™ HRP Chemiluminescent Southern Blotting Kit (Catalog No. 54-30-00) or Detector AP Chemiluminescent Blotting Kit (Catalog No. 54-30-01 or 54-30-02) OR individual detection kit components

NOTES ON...Getting Started

- Avoid contamination of reagents by using new, clean pipette tips for each pipetting operation.
- Klenow polymerase is heat labile and should be stored at –20°C. Do not store in a frost-free freezer. Keep in ice or in a portable cold unit while in use.
- Purity of the template DNA will affect the amount of labeled DNA generated. The dNTPs in the triphosphate mixture are susceptible to dephosphorylation by kinase activity, oligonucleotides are susceptible to nucleases, and Klenow polymerase can be inhibited by a variety of protein contaminants. Template DNA preparations should be as pure and free of proteins as possible.
- The buffers contained in and/or recommended for use with this kit are prepared according to the protocols listed at the end of this chapter, beginning page 20. Recipes for miscellaneous solutions can be found in the Appendix.

Detector Random Primer DNA Biotinylation At A Glance

Mix Template DNA and Random Primers
↓
Denature at 95°C
5 minutes
↓
Chill on Ice
↓
Add dNTPs and Klenow Polymerase
↓
Mix
↓
Incubate at 37°C
1 – 4 hours
↓
Stop Reaction
↓
Quantitate Probe
↓
Store Probe at -20°C

Probe Labeling by Random Priming

Steps	Critical Points
1. Dissolve 100 – 300 ng of template DNA to a final volume of 24 µL in DEPC treated water. If testing the control template DNA, add 5 µL to 19 µL of DEPC treated water.	Template purity is extremely important for the labeling efficiency using Klenow. If the template is purified from a gel, make sure to wash thoroughly in the purification procedure. Excess salts, phenolic compounds, and extraneous proteins can reduce or even inhibit Klenow activity. Use the Control Template DNA as an indicator of a pure template. If it is found that your template is reducing the labeling efficiency, additional Klenow (1-2 µL) may be added to overcome the contamination.
2. Add 20 µL 2.5X Random Primer Solution.	
3. Heat at 95°C for 5 minutes; snap cool on ice.	Denaturation of template DNA must take place to carry out any efficient labeling. The DNA/primer mix should be heated to 95°C for at least 5 minutes followed by an immediate snap cool on ice.
4. Add: 5 µL 10X dNTP Mixture 1 µL Klenow Polymerase	Subsequent dNTP additions and Klenow should follow quickly, prior to renaturation of the double stranded DNA. A-T rich templates may not label efficiently because a biotinylated dCTP is used in the incorporation by Klenow.
5. Mix gently. Pulse spin in a microcentrifuge for 3 seconds.	

Probe Labeling by Random Priming *(continued)*

Steps	Critical Points
6. Incubate at 37°C for 1 – 4 hours.	One hour is sufficient for most applications. For maximum yield of probe, allow the reaction to proceed for 4 hours. Generally, a reaction beginning with 200-300 ng template DNA generates 5- to 10-fold amplification of the template in an hour, and 10- to 50-fold amplification after 4 hours.
7. Add 5 µL Stop Buffer and mix.	
8. Proceed to Probe dilution, dot blotting and detection for quantitation.	It is imperative that the probe be quantitated. If too much probe is used in hybridization, background could occur. If too little probe is used, sensitivity may be reduced.
9. Store at –20°C until ready to quantitate and use.	See Probe Quantitation on page 18.

NOTES ON...Probe Purification:

- Following biotinylation, the newly labeled probes may be separated from unincorporated nucleotides by either ethanol precipitation or using KPL's SpinPure filters (Catalog No. 60-00-53). This is not necessary for use of probes in Southern and Northern blot detection, as unincorporated nucleotides do not significantly increase background. However, if the probes are to be used for in situ hybridization, we do recommend the removal of unincorporated nucleotides. See page 17 for Probe Purification using the SpinPure filters.

Detector™ PCR DNA Biotinylation Kit

The Detector™ PCR DNA Biotinylation Kit provides a rapid method for biotinylating DNA probes through incorporation of biotin-N_4-dCTP via a thermostable DNA polymerase in the polymerase chain reaction*.[5-7] Biotinylated probes generated using this kit are highly sensitive and allow for the identification of low copy target sequences. The process of direct labeling during PCR results in specific labeling and amplification of the sequence of interest even from crude DNA samples.

The ratio of biotin dCTP to unlabeled dCTP is optimized to produce probes with maximal biotin incorporation for detection of low copies or rare targets in mRNA and plasmid or genomic DNA. Amplification and direct labeling from small samples of genomic (1 - 100 ng) or plasmid template (10 pg - 1 ng) is most easily achieved by first optimizing the conditions for standard PCR before attempting to label the probe during the reaction.

Direct detection of a PCR biotin-labeled fragment is also possible. The biotinylated PCR product is electrophoresed, transferred to membrane and subsequently detected **without a probe** using enzyme-labeled streptavidin. A signal is then generated using the appropriate chromogenic or chemiluminescent substrate as outlined in any of the detection methods of the Detector Kits. (See Figure 6)

** Purchase of this kit does not constitute a license for PCR. A licensed polymerase and licensed thermal cycler must be used in conjunction with this product. PCR is covered by patents owned by Hoffman-La Roche, Inc. and F. Hoffman-La Roche, Inc.*

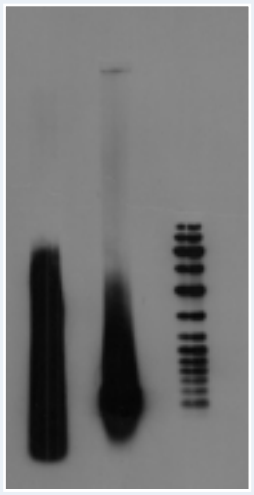


Figure 6

5 µL of random primed Lambda-HindIII (A) and 5 µL of a PCR probe (B) run on an agarose gel, transferred to Biodyne B and directly detected using the Detector AP Chemiluminescent Kit. NOTE: 5 µL is overloaded for direct detection, thus the smearing effect. Lane C is the biotinylated GeneRuler™ ladder.

The components of this kit are optimized to maximize amplification of template DNA and sensitivity of target detection.[4, 8]

Kit controls - Control primers and template DNA are included as positive controls to monitor the integrity of the assay and the components of the kit.

Probe quantitation – A biotin-labeled quantitation standard is included to quantitate the yield of biotin labeled probe. The relative concentration of probe in the hybridization buffer must be optimized for greatest sensitivity and minimal background.

- Serial dilutions of quantitation standard and newly labeled probe are fixed to positively charged nylon membrane.
- The standard and probe are detected using enzyme-labeled streptavidin, and chemiluminescent or chromogenic substrate.
- Endpoint sensitivity of the samples is compared to the standard to determine the amount of probe generated.

NOTES ON...Polymerase Selection

- DNA polymerase, 10X PCR reaction buffer and $MgCl_2$ are not included with this kit. Any thermostable polymerase that routinely and specifically amplifies the desired product with unmodified nucleotides are suitable. For complete information on individual thermostable polymerases, please refer to the specific enzyme supplier.
- This kit has been qualified with a number of thermostable polymerases including: Taq DNA Polymerase, Native or Recombinant (MBI Fermentas and Invitrogen), Taq DNA Polymerase (Promega), Taq Bead™ Hot Start Polymerase (Promega), Tth DNA Polymerase (Promega), Expand™ High Fidelity Polymerase (Roche Applied Science).

Materials and Equipment

Kit Components	Product Code	Volume
10X PCR Labeling Mix	600-0011	150 µL
Control Primers	600-0009	30 µL
Control Template DNA	600-0004	25 µL
DEPC Treated Water	50-86-03	1.0 mL
Quantitation Standard	600-0007	150 µL

Sufficient reagents are provided to perform 30 labeling reactions following the kit protocol. Reagents must be stored at -20°C and used as directed. Do not store kits in a frost-free freezer. Kit components are stable for a minimum of 1 year from date of receipt when stored as instructed.

Required Supplies and Equipment Not Included

- Licensed DNA Polymerase with 10X PCR buffer
- $MgCl_2$ stock solution
- Primers
- 1X TE (See Appendix for preparation)
- Licensed Thermal Cycler
- 20X SSC (Catalog No. 50-86-05) (See Appendix for preparation)
- Low retention or siliconized microcentrifuge tubes autoclaved, DNase free
- Microcentrifuge
- 37°C water bath or heat block
- UV Crosslinker or vacuum oven
- Nylon or nitrocellulose membrane (Biodyne® B, Catalog No. 60-00-50)
- For Probe Quantitation - Detector HRP Chemiluminescent Blotting Kit (Catalog No. 54-30-00) or Detector AP Chemiluminescent Blotting Kit (Catalog No. 54-30-01 or 54-30-02) OR individual detection kit components.

Assay Optimization

Any sequence that can be successfully amplified by PCR may be used with this protocol. It is highly recommended that the optimal conditions for PCR be determined with unmodified nucleotides for each individual template and primers before using this kit to label probe. There are many resources available for optimization of PCR. (See References 2-5.)

Optimization of the reaction should include the following considerations:

- Quality, purity and concentration of template
- PCR primer design, including melting temperature, secondary structure
- Size of desired amplimer (100 - 1100 bp is optimal for efficient labeling)
- $MgCl_2$ concentration
- Denaturation time/temperature
- Annealing duration/temperature
- Extension duration/temperature
- Enzyme concentration
- Number of cycles

Templates containing a high percentage of AT-rich sequence may label weakly as the biotin in this kit is coupled to dCTP.

NOTES ON...Getting Started

- The following protocol was designed specifically for the PCR labeling of the control template included with this kit. PCR reaction conditions should be optimized for each new template/primer set with unmodified nucleotides before use of this kit. Use the following protocol as a guideline only.
- Caution should be taken to minimize introduction of contaminating DNA and/or DNases to the PCR reaction that may result in amplification of non-specific product or no product. Always wear gloves, wash all work areas appropriately prior to beginning.
- Allow all reagents to thaw out completely, then vortex briefly and spin down in a microcentrifuge before pipetting. Keep all reagents on ice while in use *except* for the 10X Labeling Mix. Pipette reagents slowly and carefully to avoid errors.
- The buffers contained in and/or recommended for use with this kit are prepared according to the protocols listed at the end of this chapter, beginning page 20. Recipes for miscellaneous solutions can be found in the Appendix.

Detector PCR DNA Biotinylation At A Glance

Optimize PCR Conditions
With Unmodified dNTPs
↓
Prepare PCR Reaction Mix
↓
Perform PCR
~25 cycles, 40 minutes
↓
Quantitate Probe
↓
Store at -20°C

Probe Labeling by PCR

Steps

1. Prepare the reaction mix in a sterile PCR tube in order as it appears below. Place the tube on ice while pipetting.

Component	Volume	Final
DEPC Treated Water	variable	add to 50 µl
10X PCR Buffer	5 µl	1X
25 mM $MgCl_2$	4 µl	2.0 µM
10X Labeling Mix	5 µl	200 µM each
Primers	1 µl (if using the control primers)	0.5 µM each
Taq DNA Polymerase	variable	1.25 units/ 50 µl rxn
Template	1 µl (if using the control template)	1-10 ng genomic DNA or 10 pg-1 ng plasmid DNA
Total Mix	50 µl	

2. Mix the tube by tapping gently and centrifuging briefly.

Critical Points

Thaw the 10X Labeling Mix on the bench or warm it to room temperature in your hand before use. Improperly thawed dNTPs may result in a failure to produce amplification product.

By adjusting the final concentration of the 10X Labeling Mix included in the kit, probes of up to 1 kb in length may be generated. For probes less than 500 base pairs, a final concentration of 200 µM of each nucleotide is suggested (5 µL/50 µL PCR reaction), and for probes greater than 500 base pairs, 350 µM of each nucleotide is suggested (8.75 µL/50 µL PCR reaction).

Probe Labeling by PCR *(continued)*

Steps	Critical Points
3. Perform PCR according to the optimized parameters previously determined. Recommended cycling conditions for the **control** labeling reaction is as follows:	Cycling parameters will vary depending on the respective template, primers and the thermocycler used.

	Time	Temperature	Cycles
Initial Step	60 sec.	94°C	1 cycle
Denaturation	15 sec.	94°C	25 cycles
Anneal/Extend	60 sec.	68°C	25 cycles
Final Step	7 min.	72°C	
	Hold 4°C		

Gel Analysis of PCR Product

Steps	Critical Points
1. Load approximately 5 µl of sample on a 0.8-1% TBE or TAE agarose gel.	The PCR product is evaluated by agarose gel electrophoresis to verify expected product size and to estimate the overall yield of the reaction. If the control DNA template is amplified, a 500 bp product is expected.
2. Electrophorese according to the equipment manufacturer's recommendations. Stain to visualize product size verification.	
3. Transfer the remaining aliquot of sample to a siliconized tube and store at 2-8°C until quantitation.	See Probe Quantitation on page 18.

NOTES ON…Removal of non-specific amplimers, primers and unincorporated nucleotides

- Following biotinylation and analysis of the product on an agarose gel, separation of the newly labeled probe from unincorporated nucleotides, primers or non-specific amplimers may be desired if using the labeled product in hybridization assays. This is usually not necessary for membrane applications, as unincorporated nucleotides do not significantly increase background. If non-specific PCR products are evident in the final reaction, excise the correct band from an agarose gel and subsequently purify the product using well-established methods.
- If probes are to be used in *in situ* applications, the use of Spin-Pure filters (Catalog No. 60-00-53) is recommended. The use of 5' → 3' Select B columns, which have been developed specifically for purification of biotinylated probes, is also recommended. G50 Microspin Purification Columns should not be used because biotin sticks to the resin resulting in loss of labeled PCR product.

Detector RNA *in vitro* Transcription Biotinylation Kit

The Detector™ RNA *in vitro* Transcription Biotinylation Kit provides a method for synthesizing biotin labeled RNA probes by *in vitro* transcription through incorporation of biotin UTP. Strand-specific probes may be generated using either the T7 or SP6 RNA polymerase.[9] The transcript reaction generates full length, single-stranded RNA probes that can be used in a variety of applications, including membrane and *in situ* hybridization. The incorporation of biotin UTP by SP6 or T7 polymerase is very efficient, resulting in "hot" labeled RNA probes. These non-isotopic biotin labeled RNA probes are stable for at least one year. Single-stranded RNA probes hybridize more effectively to target molecules because they do not self-hybridize as DNA probes do.[10] RNA probes offer greater sensitivity than DNA probes because RNA-RNA or RNA-DNA hybrids are more stable than DNA-DNA duplexes in hybridization.

Direct detection of a biotin-labeled RNA probe is also possible. The biotinylated transcript can be electrophoresed, transferred to a membrane, and subsequently detected **without a probe** using fluorochrome-labeled streptavidin or enzyme-labeled streptavidin and a chromogenic or chemiluminescent substrate as directed in any of the detection methods of the Detector kits.

The components of this kit are optimized to maximize the synthesis of RNA as well as the sensitivity of target detection.

Template requirements – In order to generate single stranded RNA probes, you must begin with a DNA template with SP6 or T7 promoter sequences upstream from the desired template sequence. Two methods to prepare these types of templates are recommended and further detailed in the protocol:

- Cloning the DNA into a vector with the SP6 and T7 promoter sequences on either side of the cloning site
- PCR of the DNA template with the promoter sequences built into the primers.

Kit control – A human ß-Actin DNA template is included in this kit to serve a two-fold purpose: 1) to act as a control for the integrity of the kit components, and 2) to generate a control probe for detection of ß-actin on human or mouse Northern blots. Synthesized in a strand-specific manner, an antisense transcript may be generated using T7 RNA polymerase and a sense transcript may be generated using SP6 RNA polymerase. The expected size of either transcript is ~400 bases. If the control probe is to be used in a Northern blot, the anti-sense (T7) probe must be used.

Probe quantitation – The second kit control, the Biotinylated Quantitation Standard is used to quantitate the yield of biotin-labeled RNA probe.

NOTES ON…Getting Started

- All tubes and pipet tips should be autoclaved or purchased as RNase free prior to working with RNA.
- All glassware and equipment used should be RNase free.
- Always wear gloves when working with RNA because human skin contains abundant amounts of RNases.

Quantitation of the probe is essential as the concentration of probe used in the hybridization reaction is critical for greatest sensitivity and minimal background on a membrane.

- Serial dilutions of quantitation standard and newly labeled probe are fixed to positively charged nylon membrane.
- The standard and probe are detected using enzyme-labeled streptavidin, and chemiluminescent or chromogenic substrate.
- Endpoint sensitivity of the samples is compared to the standard to determine the relative amount of probe generated.

Probe storage – Stable for at least one year when stored at -20°C. Because biotinylated probes stick to normal microcentrifuge tubes, it is recommended that probes be stored in low retention or siliconized tubes.

Materials and Equipment

Kit Components	Product Code	Volume
10X Ribonucleotide Labeling Mix	600-0013	60 µL
10X Transcription Buffer	600-0010	60 µL
ß-Actin Template	600-0017	16 µL
RNase Inhibitor	600-0012	13 µL
SP6 RNA Polymerase	600-0015	20 µL
T7 RNA Polymerase	600-0014	20 µL
DNase I	600-0016	20 µL
DEPC Treated Water	50-86-03	1.0 mL
Quantitation Standard	600-0007	150 µL
Spin-Pure Filters	60-00-53	5 filters

Sufficient reagents are provided to perform 20 labeling reactions following the protocol provided in this manual. All reagents must be stored at -20°C except for the Spin-Pure Filters that should be stored at room temperature. Do not store kits in a frost-free freezer. Kit components are stable for a minimum of 1 year from date of receipt when stored as instructed.

Detector RNA *in vitro* Transcription Biotinylation At A Glance

Preparation of DNA Template
↓
Prepare Labeling reaction mix
↓
Incubate at 37°C
2 hours
↓
Add DNase I
↓
Incubate at 37°C
15 minutes
↓
Quantitate Probe
↓
Store at –20°C
[or -70°C for long-term storage]

Required Supplies and Equipment Not Included

- Low retention or siliconized microcentrifuge tubes
- Microcentrifuge
- 37°C water bath or heat block
- 0.1 M DTT
- 20X SSC (Catalog No. 50-86-05 or see buffer prep section)
- Positively charged nylon membrane (Biodyne B, Catalog No. 60-00-50)
- UV Crosslinker or vacuum oven
- For Probe Quantitation - Detector HRP Chemiluminescent Blotting Kit (Catalog No. 54-30-00) or Detector AP Chemiluminescent Blotting Kit (Catalog No. 54-30-01 or 54-30-02) OR individual detection kit components

NOTES ON...DNA Template Preparation

Templates Cloned in Vectors Containing SP6 or T7 Promoters

To generate template by this method, it is necessary to first place the desired double stranded DNA sequence under the control of either the SP6 or T7 promoter. A variety of commercially available vectors have been constructed to place the SP6 and T7 RNA promoter sequences opposite each other on either side of the multiple cloning site. To prepare the template for transcription, linearize the plasmid using the appropriate restriction enzyme such that transcription is initiated at the appropriate promoter and is terminated close to the end of the desired sequence. It is important that the restriction digestion be performed to completion to eliminate undigested plasmid DNA, which can give rise to transcripts containing extraneous sequence. It is also important to digest with an enzyme that leaves a 5´ overhang or blunt end. Enzymes that cleave leaving a 3´ overhang may give rise to aberrant transcripts other than what is expected.

Following the restriction enzyme digestion, either ethanol precipitate or filter purify the template using the Spin-Pure filters provided in this kit. Resuspend the linear DNA in TE Buffer (See Solution Preparation) at a concentration of 0.25 - 1.0 µg/µl. See specific section for use of Spin-Pure filters.

PCR Generated Template

Alternatively, PCR may be used to amplify the DNA fragment flanked by the SP6 and/or T7 promoter sequences. Using this method, either antisense or sense RNA transcripts can be generated from a single construct. Design PCR primers that include the probe specific sequence appended to the 3´ end of the promoter sequence. To place the SP6 or T7 primer at the 5´ end of the probe specific sequence, design the oligonucleotide to include the SP6 or T7 sequence listed below, followed at the 3´ end by the sense (coding) strand of the probe specific sequence reading in the 5´ to 3´ direction. To place the SP6 or T7 primer at the 3´ end of the probe specific sequence, design the oligonucleotide to include the SP6 or T7 sequence listed below, followed at the 3´ end by the antisense (noncoding) strand of the probe specific sequence reading in the 3´ to 5´ direction. The primers should contain between 6 - 10 bases of probe-specific sequence.

SP6 Promoter: 5´ ACG ATT TAG GTG ACA CTA TAG AA 3´
T7 Promoter: 5´ AGT TAA TAC GAC TCA CTA TAG GGA 3´

The PCR product can be used in the transcription reaction without further purification. It is necessary, however, to determine the concentration of the PCR product and verify the expected size of the PCR product before performing transcription.

Make sure antisense probes are prepared because sense probes will not hybridize to the mRNA.

Probe Labeling by *in vitro* Transcription

Steps	Critical Points
1. Prepare a fresh tube of 0.1 M DTT (see buffer prep section)	All tubes and tips should be autoclaved prior to working with RNA. It is also essential that any glassware or equipment used be RNase free. Always wear gloves when working with RNA as human skin contains abundant amounts of RNases.
2. Prepare the reaction mix in a DNase/RNase-free microcentrifuge tube at room temperature in the following order:	Place T7 and SP6 RNA Polymerases on ice. Allow all other reagents to thaw completely on the bench. This is especially important for the 10X Transcription Buffer. Vortex reagents briefly and spin down in a microcentrifuge for a few seconds. Place everything except for the 10X Transcription Buffer on ice.

Component	Volume	Final
DEPC Treated Water	variable	add to 30 µL
RNase Inhibitor	0.5 mL	20 units
Linearized DNA Template (use 2 µL for control DNA)	variable	1 µg
10X Labeling Mix	3 µL	1X
10X Transcription Buffer	3 µL	1X
0.1M DTT (buffer prep section)	3 µL	0.01 M
Polymerase (SP6 or T7)	1 µL	50 units/µL
Total Volume	30 µL	

Keep the 10X Transcription Buffer at room temperature during use. Use of cold 10X Transcription Buffer in the labeling reaction may cause precipitation of the DNA template and could result in a low yield of transcript.

Probe Labeling by *in vitro* Transcription *(continued)*

Steps	Critical Points
3. Mix the tube by flicking gently and centrifuge briefly.	
4. Place the tube at 37°C for 2 hours.	
5. Add 1 µL of DNase I, flick the tube gently, and centrifuge briefly.	
6. Incubate 37°C for 15 minutes.	
7. Place the tube on ice or store at –20°C until needed for quantitation and analysis.	Because of the high concentration of the probe and its susceptibility to RNases, aliquoting of the probe for storage is recommended. For long term storage, freeze at -70°C.

NOTES ON... Probe Purification:

- Following biotinylation, the newly labeled probes may be separated from unincorporated nucleotides by either ethanol precipitation or using KPL's SpinPure filters (Catalog No. 60-00-53). This is not necessary for use of probes in Southern and Northern blot detection, as unincorporated nucleotides do not significantly increase background. However, if the probes are to be used for in situ hybridization, we do recommend the removal of unincorporated nucleotides. See below for Probe Purification using the SpinPure filters.

Probe Purification *(optional)*

Steps	Critical Points
1. Ensure that the sample reservoir is firmly placed into the filtrate receiver.	
2. Add 1XTE to the probe to increase the volume. Pipette 50 - 500 µL of the sample into the sample reservoir. Cap the Spin-Pure filter and place into a microcentrifuge.	Do not tear the filter with a pipet tip.
3. Centrifuge at 5,000 x g for 15 minutes at room temperature. Continue centrifugation until filter is dry (a volume of 500 µL can usually be concentrated in 20 minutes).	
4. If removing primers and nucleotides from amplified product, centrifuge at 14,000 x g.	
5. Recover sample from the filter with DEPC-treated water or 1X TE by rinsing the surface. Highest yields result from two rinses of 20 µL each.	

Gel Analysis of Transcript

Steps	Critical Points
1. Pre-heat the probe to 68°C for 5 minutes.	The expected size of the transcript will be somewhat different when comparing an RNA probe to a DNA marker lane, but an approximate size estimate can still be determined.
2. Run 2-5 µL of the probe on either a 1X TBE agarose gel or a formaldehyde gel containing 0.5 µg/mL ethidium bromide. Include DNA or RNA markers on the gel for proper size.	Biotinylated transcripts run larger than their unbiotinylated counterparts.
3. Transfer the remaining aliquot of sample to a siliconized tube and store at 2–8ºC until quantitation.	See Probe Quantitation on page 18.

Probe Quantitation

As stated earlier, quantitation of the newly biotinylated nucleic acid probe is essential to the success of non-isotopic detection, as the concentration of probe used in the hybridization reaction is critical for greatest sensitivity with minimal background. For optimal use, the probe must be quantitated for biotin incorporation, not a spectrophotometric reading. The amount of biotinylated probe synthesized is influenced by:

- Template purity
- Template concentration
- Incubation time

The following protocol allows for the determination of the amount of biotinylated DNA or RNA synthesized from the respective labeling reaction. This is accomplished through dot blot of diluted probe and determination of relative specific activity (RSA) as compared to the Quantitation Standard provided in each kit. The Quantitation Standard is provided at a known concentration of 50 ng/µL. This is a relative concentration based on the amount of biotin detected. RSA of the labeled probe is defined by the relative amount of biotin incorporated during labeling. Biotinylated probes need only be quantitated once; they do not lose activity over time.

Probe Dilution and Dot Blotting

Steps	Critical Points
1. Dilute probe 1/200 (1 µL probe to 199 µL 1X TE)	Dilute the probe in 1X TE if using low retention tubes. If low retention tubes are not available, use 2X SSC/0.1% SDS as the diluent. Biotin may stick to the standard microcentrifuge tubes, reducing the concentration of the probe.
2. Prepare a 2-fold serial dilution panel from the 1/200 dilution of probe. (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, and 1/512)	Example of 2 fold dilution: Add 10 µL of diluent to 10 tubes. Add 10 µL of the 1/200 dilution to the diluent in tube 1 and mix. Remove 10 µL from tube 1 and add it to tube 2 and mix. Repeat for the entire panel.
3. Prepare a 2-fold serial dilution panel from the Quantitation Standard as described above. Do **NOT** prepare an initial 1/200 dilution.	The Quantitation Standard is provided at a set relative specific activity level. No dilution is required prior to the two-fold series.
4. Spot 1 µL of each dilution onto a dry, pretreated membrane starting with the 1/2 dilution of each sample. Use the picture on page 19 as a guide.	Membrane Pre-Treatment - Grid the membrane with a pencil if desired. Wet membrane in molecular grade water followed by a 10-minute wash in 5X SSC. Dry the membrane completely before use. Pretreatment need only be performed one time and may be stored for future use.
5. UV crosslink the damp spotted membrane according to manufacturer's instructions.	
6. Dry the membrane for at least 10 minutes.	
7. Detect dots as described below.	

Probe Quantitation Detection Using AP–Streptavidin and CDP–Star

See Buffer Preparation Section for buffer details. All steps are to be carried out at room temperature with gentle agitation or rocking.

1. Incubate the spotted membrane in Blocking Solution for 30 minutes.
2. Incubate the membrane for 30 minutes in fresh Blocking Solution with AP-SA diluted 1:10,000.
3. Wash the membrane in 1X Phosphatase Wash Solution 3 times for 5 minutes each.
4. Rinse the membrane 2 times for 2 minutes each in 1X Assay Buffer.
5. Incubate membrane for 5 minutes in CDP-Star substrate. Blot membrane on filter paper to remove excess substrate. Place membrane in a hyb bag or between sheet protectors and expose to X-ray film for a target of 2 minutes – 10 minutes.
6. Proceed to probe RSA determination section.

Probe Quantitation Detection Using HRP-Streptavidin and LumiGLO

See Buffer Preparation Section for buffer details. All steps are to be carried out at room temperature with gentle agitation or rocking.

1. Incubate spotted membrane in Blocking Solution for 30 minutes.
2. Incubate membrane for 20 minutes in fresh Blocking Solution with HRP-SA diluted 1:500.
3. Wash the membrane in 1X Biotin Wash Solution 3 times 5 minutes each.
4. Incubate membrane for 1 minute in LumiGLO solution. Blot membrane on filter paper to remove excess substrate. Place membrane in a hyb bag or between sheet protectors and expose to X-ray film for a target of 10 minutes.
5. Proceed to probe RSA determination section.

Determination of Probe Relative Specific Activity (RSA)

Steps

1. Calculate ratio of probe to standard (P/S):
   P/S = probe endpoint dilution ÷ standard endpoint dilution
2. Calculate probe RSA:
   Probe RSA = standard RSA x P/S

Examples: (See Figure)

Probe 1 (P1) endpoint dilution = 1/16
Standard (S) endpoint dilution = 1/32

1. P/S = 1/32=1/2

2. Probe RSA = 50ng/µL x 1/2 = 25 ng/µL

Probe 2 (P2) endpoint dilution = 1/64

Standard (S) endpoint dilution = 1/32

1. P/S = 64/32 = 2

2. Probe RSA = 50 ng/µL x 4 = 100 ng/µL

EXPECTED PROBE YIELD
Total yield = probe RSA x 55 µL (reaction volume)

Critical Point

Do not factor in the initial 1/200 dilution of probe, as the standard curve concentration has already been adjusted.

Quantitation of DNA Probe

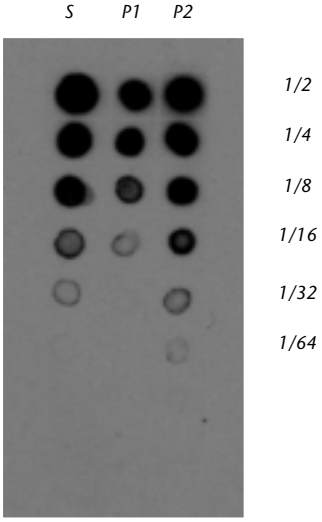


Figure 7

Steps

Examples: (See Figure)

Probe 1 (P1) endpoint dilution = 1/1024
Standard (S) endpoint dilution = 1/16

1. 1024/16

2. Probe RSA = 50 ng/µL x 64 = 3200 ng/µL

(P1) endpoint dilution 1/1024

(S) endpoint dilution 1/16

P/S = 1024/16 = 64
50 ng/mL x 64 = 3200 µg/µL

EXPECTED PROBE YIELD
Total yield = probe RSA x 55 µL (reaction volume)

Critical Point

Do not factor in the initial 1/200 dilution of probe, as the standard curve concentration has already been adjusted.

Quantitation of RNA Probe

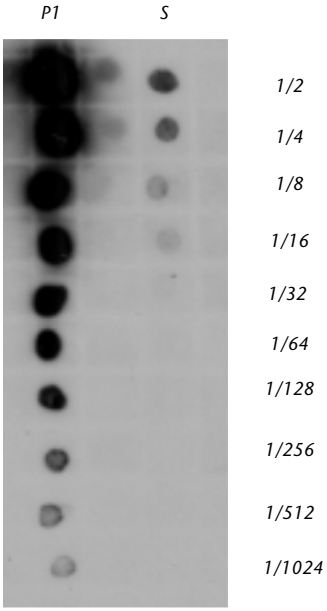


Figure 8

Probe Storage and Reuse:

- Once the probe is made and quantitated, it can be stored in the freezer for at least one year. The probe does not need to be requantitated.
- Probes used in hybridization reactions may be reused. Simply store the hybridization solution containing the probe in a polypropylene conical tube at 2-8°C (formamide-based hybridization solutions) or –20°C (aqueous-based hybridization solutions) until future use.
- The probes may be denatured again by heating the solution to 68°C for 10 minutes (formamide based solutions) or boiling (aqueous based solutions) for 10 minutes before use.

Buffer Preparation

The following procedures allow for the preparation of the various buffers supplied with and/or recommended for use with the Detector Labeling Kits – Random Primer DNA Biotinylation, PCR DNA Biotinylation, and RNA *in vitro* Transcription Biotinylation. Additional buffer recipes can also be found in the Appendix, Chapter 7.

Steps	Critical Points
Detector Block Solution – to be prepared fresh daily	
1. Based on the total desired 1X Detector Block volume, weigh out 0.2% - 1% w/v Detector Block Powder. For detection with AP and CDP-Star, use 0.2% and for detection with HRP and LumiGLO, use 1%. (.2-1g of Detector Block Powder per 100 mL of 1X Solution)	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. Dilute the solution with 1:5 v/v 5X Detector Block Solution. Ex., for 50 mL of 1X Detector Block: Detector Block Powder 0.1 g (AP/CDP-Star detection only) or 0.5 g (HRP/LumiGLO detection only) 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 that may occur as a result of unsolubilized powder. The amount of powder used can be increased to decrease background. However, too much powder may reduce sensitivity.
Biotin Wash Solution (HRP and LumiGLO detection only)	
1. Dilute 1 part 10X Biotin Wash to 9 parts molecular biology grade water. Mix well.	Biotin Wash Solution may be made to 1X in bulk and stored for 1-3 months on the bench.
Phosphatase Wash Solution (AP and CDP-Star detection only)	
1. Dilute 1 part 5X Phosphatase Wash to 4 parts molecular grade water. Mix well.	SDS may fall out of solution in the 5X concentrate. Simply place the bottle in a 37°C – 65°C water bath for a few minutes and mix until the solution is homogenous before diluting.
LumiGLO – to be prepared fresh before its use (HRP and LumiGLO detection only)	
1. Mix together 1 part LumiGLO A to 1 part LumiGLO B.	
Phosphatase Assay Buffer (AP and CDP-Star detection only)	
1. Dilute 1 part 10X Assay Buffer to 9 parts molecular biology grade water. Mix well.	

References

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[2] Feinberg, A. P., and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132: 6-13.

[3] Feinberg, A. P., and Vogelstein, B. (1984) Addendum to: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 137:266-267.

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Southern Blotting with Detector™ Chemiluminescent Blotting Kits

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.

KPL provides two methods for the non-radioactive detection of Southern blots. Each relies on a different enzyme and substrate system, one being alkaline phosphatase/CDP-Star, Chemiluminescent Substrate (Detector AP Chemiluminescent Blotting Kit, 54-30-01/02) and the other horseradish peroxidase/LumiGLO, Chemiluminescent Substrate (Detector HRP Chemiluminescent Blotting Kit, 54-30-00). As such, a variety of DNA samples in a number of applications can be quickly detected.

Kit selection will depend on the requirements of the assay. Due to its high sensitivity, the 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

Detector Southern Blotting At A Glance

Gel Electrophoresis
↓
Alkaline Transfer
↓
Hybridization and Detection

AP Chemiluminescent Blotting	**HRP Chemiluminescent Blotting**
Prehybridization	**Prehybridization**
1 hour	1 hour
↓	↓
Hybridization	**Hybridization**
3-18 hours	3-16 hours
↓	↓
2X SSPE/0.1% SDS washes	**0.5X SSPE**
2 X 15 min	2 X 10 min
↓	↓
0.2X SSPE/0.1% SDS washes	**0.5X SSPE elevated temp.**
2 X 15 min	2 X 10 min
↓	↓
2X SSPE wash	**Detector Block**
5 min	30 min
↓	↓
Detector Block	**HRP-Streptavidin**
45 min	20 min
↓	↓
AP-Streptavidin	**Biotin Wash Solution**
30 min	3 X 5 min
↓	↓
Phosphatase Wash Solution	**LumiGLO**
3 X 5 min	1 min
↓	↓
Phosphatase Assay Buffer	**Film Exposure**
2 X 2 min	10 min-2 hours
↓	
CDP-Star	
5 min	
↓	
Film Exposure	
2 min - 2 hours	

also suitable for rapid detection of plasmid DNA, PCR products and dot blots. Because of the kinetics of CDP-Star, multiple exposures of the blot may be performed over a period of several days.

While not as sensitive as the alkaline phosphatase/CDP-Star method, the Detector HRP Chemiluminescent Blotting Kit offers a cost-effective alternative to non-radioactive Southern blotting that continues to yield results comparable to ^{32}P in far less time. Sub-picogram detection can be achieved in a 15-minute film exposure. The Detector HRP Chemiluminescent Blotting Kit is designed for routine Southern blotting applications including analysis of plasmid DNA on membrane. This kit has also been shown to be the better approach for bacterial colony lift hybridization and plaque hybridization. (See Chapter 7 - Appendix: Miscellaneous Applications, page 55) Where sensitivity is not an issue, LumiGLO Chemiluminescent Substrate may be replaced by TMB Membrane Substrate (Catalog No. 50-77-03) for colorimetric detection that saves time and expense of using film and related equipment. This may be particularly useful for control experiments because variability due to film exposure is eliminated.

Both kits were developed and optimized with Pall Biodyne, B Nylon Membrane for the best signal-to-noise ratio. Exposure to X-ray film produces a permanent record of chemiluminescent emissions. Detection may also be accomplished using a chemiluminescent imaging system. While there are a variety of Southern blotting procedures, the following protocols are recommended when using the respective Detector kit to deliver the greatest sensitivity without background.

Materials and Equipment

Detector AP Chemiluminescent Blotting Kit

	Catalog No. 54-30-01 2000 cm²		Catalog No. 54-30-02 500 cm²	
Kit Components	**Product Code**	**Volume**	**Product Code**	**Volume**
Formamide Hybridization Buffer	50-86-12	200 mL	50-86-11	50 mL
Detector Block Solution (5X)	71-83-02	2 x 240 mL	71-83-01	120 mL
Detector Block Powder	72-01-03	10 g	72-01-02	10 g
Phosphatase-labeled Streptavidin (AP-SA)	475-3001	0.1 mL	475-3001	0.1 mL
Phosphatase Wash Solution Concentrate (5X)	50-63-17 50-63-11	1000 mL 200 mL	50-63-11 50-63-18	200 mL 100 mL
Phosphatase Assay Buffer (10X)	50-63-12	200 mL	50-63-13	50 mL
CDP-Star Chemiluminescent Substrate	50-60-03	100 mL	50-60-04	30 mL

Sufficient reagents are provided with Catalog No. 54-30-01and Catalog No. 54-30-02 to test approximately 2000 cm² of membrane (20 each of 10 cm x 10 cm blots) and 500 cm² of membrane (5 each of 10 cm x 10 cm blots), respectively.

Detector HRP Chemiluminescent Blotting Kit

	Catalog No. 54-30-00 2000 cm²	
Kit Components	**Product Code**	**Volume**
Formamide Hybridization Buffer	50-86-09	120 mL
Detector Block Solution (5X)	71-83-01	120 mL
Detector Block Powder	72-01-01	10 g
Peroxidase-labeled Streptavidin (HRP-SA)	474-3003	1 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 with Catalog No. 54-30-00 to test approximately 2000 cm² of membrane (20 each of 10 cm x 10 cm blots).

Required Supplies and Equipment Not Included in Detector Kits

- Biotin-labeled nucleic acid probe
- Herring Sperm DNA (Catalog No. 60-00-14)
- Ethidium bromide
- 20X SSPE (See Appendix for preparation)
- SDS
- UV Transilluminator
- UV crosslinker or vacuum oven
- Capillary transfer apparatus
- Waterbath or hybridization oven
- Heat-sealed hybridization bags (Catalog No. 60-00-51) or hybridization bottles
- X-ray film and film cassettes or a chemiluminescent imaging system
- Positively charged nylon membrane (Biodyne B, Catalog No. 60-00-50)
- Molecular biology grade water
- Agarose
- Horizontal electrophoresis tank
- 20X SSC (Catalog No. 50-86-05 or see Appendix for preparation)
- NaOH stock solution

NOTES ON…Storage Conditions

- The 5X Phosphatase Wash Solution Concentrate should be removed from the Detector AP Chemiluminescent Blotting Kit and stored at room temperature. All other components should be stored at 2-8°C.
- For optimal performance, Formamide Hybridization Buffer should be warmed (37-50°C) and aliquotted into DNase/RNase free tubes and then stored at 2-8°C. Do not store Formamide Hybridization Buffer at -20°C. Prolonged storage of this buffer at -20°C may result in decreased sensitivity.
- Reagents are stable for a minimum of one year when stored as instructed.

NOTES ON…Warnings and Precautions

- Read ALL instructions thoroughly before using these kits.
- 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.

Gel Electrophoresis

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. The gel should not be thicker than 6 mm for efficient transfer.
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 and the molecular weight of the detected band later.	
4. Immediately continue with the transfer.	

Alkaline Transfer

Alkaline transfer is highly recommended for transfer of DNA onto positively charged nylon membrane. This method is more reproducible than high salt overnight transfers or any other 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. 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 <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/1.5 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.	
7. After transfer, neutralize and rinse the membrane for 5 min in a generous amount of 5X SSC. Place membrane on filter paper 2-4 min and fix the DNA to the membrane using a UV crosslinker or vacuum oven according to manufacturer's instructions.	Most manufacturers claim that nucleic acid transferred to positively charged membrane does not need to be fixed; however, we have found that sensitivity is greatest after cross-linking or baking.
8. Store membranes between two pieces of blotting paper and seal in a hybridization bag. Store bag in a cool and dry place until detection is performed.	See Hybridization and Detection Procedures: AP, pages 27-28; HRP, pages 29-30

NOTES 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 AP Chemiluminescent Blotting Kit Protocol:

Prehybrization and Hybridization

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 0.1 mL/ cm^2 of membrane is recommended. Use the guidelines listed at the right.	Use 10 mL of Formamide Hybridization Buffer per 100 cm2 membrane. (For example: a 10 cm X 10 cm membrane = 100 cm^2 = 10 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 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. For convenience, 10 mL of Hybridization Buffer regardless of blot size may be used. The hybridization buffer containing probe may be reused, and 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 1 mL of Formamide Hybridization Buffer that is used. If using KPL's Herring sperm DNA, add 10 µL per mL of 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. **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.
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 directly to the Prehybridization Buffer at 50 ng per mL of Buffer.	Pipet the probe directly into the buffer and not directly on the blot.
8. Incubate the membrane with gentle agitation for 16-18 hours at 42°C.	The desired hybridization temperature when using DNA probes is 42°C. Shorter hybridization time (3 hours) is possible for detection of abundant target.
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.	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.

Prehybrization and Hybridization *(continued)*

Steps	Critical Points
10. Remove the membrane from the Hybridization buffer. Wash 2 X 15 minutes in a generous volume (at least 1 ml per cm^2 of membrane) of 2X SSPE/0.1% SDS 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 15 minutes at the elevated temperature in the temperature equilibrated 0.2X SSPE/0.1% SDS wash.	Completely cover the membrane with the wash solution.
12. Perform the final wash in 2X 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 with AP-SA and CDP-Star

Steps	Critical Points
1. Prepare enough 1X Detector Block blocking/ diluent solution for the blocking step and for the AP-SA conjugate dilution.	Use at least 0.25 mL 1X blocking/diluent solution per cm^2 membrane (*i.e.*, 10 cm x 10 cm = 100 cm^2 = 25 mL blocking/diluent solution per incubation). See Buffer Preparation Section for instructions on preparation of 1X Detector Block. Note that the concentration of Detector Block Powder is dependent on the enzyme used for detection. Solution preparation for use with AP-SA systems differs from that used with HRP-SA systems.
2. Incubate Southern blot with 1X 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.** 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 AP-SA conjugate at 1:10,000 in fresh 1X Detector Block (2.5 µL conjugate + 25 mL blocking solution). Mix well.	Use at least 0.25 mL of diluted conjugate per cm^2 membrane.
4. Pour off the blocking/diluent solution (from step 2) from the membrane and add the diluted AP-SA solution. Incubate for 30 minutes.	
5. Place membrane in a clean container. Wash the membrane in 1X Phosphatase Wash Solution 3 times for 5 minutes each.	See Buffer Preparation Section for instructions on preparation of 1X Phosphatase Wash Solution. Use at least 0.75 mL/cm^2.
6. Rinse the membrane 2 times for 2 minutes each in 1X Phosphatase 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. See Buffer Preparation Section for instructions on preparation of 1X Phosphatase Wash Solution. Use at least 0.4 mL/cm^2.
7. Incubate membrane for 5 minutes in CDP-Star, Chemiluminescent Substrate. Blot membrane on filter paper to remove excess substrate. Place membrane in a hyb bag or between sheet protectors and expose to X-ray film for an initial exposure of 2 minutes. Adjust exposure time for optimal signal-to-noise ratio.	Use 0.05 mL CDP-Star per cm^2 of membrane (*i.e.,* 10 cm x 10 cm = 100 cm^2 = 5 mL CDP-Star). Gently pipet the substrate directly on the membrane so that the substrate remains on the membrane, not in the tray. Do not agitate or rock the membrane.
8. Develop film either manually or by using a mechanical processor.	

NOTES ON…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 to 4 hours persisting 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.

Detector HRP Chemiluminescent Blotting Kit Protocol:

Prehybrization and Hybridization

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 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. 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 1 mL of Formamide Hybridization Buffer that is used. If using KPL's Herring Sperm DNA, add 10 µL per mL of 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. **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.
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.	Pipet the probe directly into the buffer and not 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.

Prehybrization and Hybridization *(continued)*

Steps	Critical Points
9. Prepare post-hybridization washes: 0.5X SSPE. Place 1/2 of the wash solution at room temperature. The other half should be equilibrated to 55ºC at least 2 hours prior to use.	Washes may be modified to contain different concentrations of SSPE 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 Hybridization buffer. Wash 2 X 10 minutes in a generous volume (at least 1 ml per cm^2 of membrane) of 0.5X 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 55°C in the temperature equilibrated 0.5X SSPE wash.	Completely cover the membrane with the wash solution.
12. Continue immediately with detection.	Never allow membrane to dry out during hybridization and detection.

Detection with HRP-SA and LumiGLO

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 Section for instructions on preparation of 1X Detector Block. Note that the concentration of Detector Block Powder is dependent on the enzyme used for detection. Solution preparation for use with AP-SA systems differs from that used with HRP-SA systems.
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 blocking solution). Mix well.	Use at least 0.3 mL of diluted conjugate solution per cm^2 membrane.
4. Pour off the blocking/diluent solution (from step 2) from the membrane and add the diluted HRP-SA solution. Incubate for 30 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 Section 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°C.
7. Incubate membrane for 1 minute in LumiGLO Chemiluminescent Substrate. Blot membrane on filter paper to remove excess substrate. Place membrane in a hyb 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.	
8. Develop film either manually or by using a mechanical processor.	

NOTES ON…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 case dark spots to appear on the film.

Stripping and Reprobing a Southern Blot

Membranes hybridized with biotinylated probes can be stripped and reprobed after detection with either CDP-Star or LumiGLO provided the membrane is never allowed to dry prior to stripping (i.e., store membrane in a covered container in 1X SSPE).

Stripping a DNA Probe

1. Wash membrane in 1X 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 membrane 2 times for 5 minutes each in 2X SSC.
4. Reprobe immediately or store stripped membrane dry at room temperature or 4°C.

Buffer Preparation

Sufficient reagents are provided in both the Detector™ AP and HRP Chemiluminescent Blotting Kits 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 in Southern blotting using this system, recommended preparation of kit buffers is detailed below. Required solutions not provided with the kit can be found in the Appendix, page 68.

Solutions from Detector AP Chemiluminescent Blotting Kit

Steps	Critical Points
1X Detector™ Block Solution– to be prepared fresh daily	
1. Based on the total desired 1X Detector Block volume, weigh out 0.2% w/v Detector Block Powder **for detection with AP and CDP-Star.**	If the block solution is not prepared daily, sensitivity could be reduced and background can 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. (Approximately 30 seconds to 1 minute)	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. Insure that all 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. Do not add the powder to the entire volume of the 1X Detection Block Solution. This will result in clumps that will be hard to solubilize.
3. Dilute the solution with 1:5 v/v 5X Detector Block Solution. Example, for 50 mL of 1X Detector Block: Detector Block Powder 0.1 g Molecular Biology Grade H_20 40 mL 5X Detector Block Solution 10 mL	

Solutions from Detector AP Chemiluminescent Blotting Kit *(continued)*

Steps	Critical Points
Phosphatase Wash Solution	
1. Dilute 1 part 5X 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 prior to use.
Phosphatase Assay Buffer	
1. Dilute 1 part 10X Assay Buffer with 9 parts molecular biology grade water. Mix well.	

Solutions from HRP Chemiluminescent Blotting Kit

Steps	Critical Points
1X Detector™ Block Solution – to be prepared fresh daily	
1. Based on the total desired 1X Detector Block volume, weigh out 1% 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 can 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. (Approximately 30 seconds to 1 minute)	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. Insure that all 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. Do not add the powder to the entire volume of the 1X Detection Block Solution. This will result in clumps that will be hard to solubilize.
3. 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	
1X Biotin Wash Solution	
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.	

Northern Blotting with Detector AP Chemiluminescent Blotting Kit

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 and cells. The comparison of these levels to control samples gives insight to relative expression of genes and the impact of various factors on that expression.

As noted in earlier discussion, the development of improved substrates has provided the means by which even rare messages can now be detected in total RNA samples. Chemiluminescent detection using CDP-Star substrate allows greater sensitivity and permits faster exposure time than peroxidase-based substrates. For this reason, the Detector™ AP Chemiluminescent Blotting Kit takes advantage of the superior performance of CDP-Star to offer a system that surpasses other non-radioactive methods for the analysis of mRNA by Northern blotting.

Total RNA samples immobilized on solid supports such as nylon membrane are prehybridized, then hybridized with biotinylated DNA or RNA probes. The membrane is washed to remove excess probe and blocked to prevent non-specific binding of a reporter conjugate. 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 and incubated with CDP-Star Chemiluminescent Substrate.

The Detector AP Chemiluminescent Blotting Kit was developed and optimized with Pall Biodyne B Nylon Membrane for remarkably high sensitivity and low background. Exposure to X-ray film produces a permanent record of chemiluminescent emissions. Detection may also be accomplished using a chemiluminescent imaging system. 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 when using the Detector system.

Materials and Equipment

	Catalog No. 54-30-01 2000 cm²		Catalog No. 54-30-02 500 cm²	
Kit Components	**Product Code**	**Volume**	**Product Code**	**Volume**
Formamide Hybridization Buffer	50-86-12	200 mL	50-86-11	50 mL
Detector Block Solution (5X)	71-83-02	2 x 240 mL	71-83-01	120 mL
Detector Block Powder	72-01-03	10 g	72-01-03	10 g
Phosphatase-labeled Streptavidin (AP-SA)	475-3001	0.1 mL	475-3001	0.1 mL
Phosphatase Wash Solution Concentrate (5X)	50-63-17 50-63-11	1000 mL 200 mL	50-63-11 50-63-18	200 mL 100 mL
Phosphatase Assay Buffer (10X)	50-63-12	200 mL	50-63-13	50 mL
CDP-Star Chemiluminescent Substrate	50-60-03	100 mL	50-60-04	30 mL

Sufficient reagents are provided with Catalog No. 54-30-01and Catalog No. 54-30-02 to test approximately 2000 cm² of membrane (20 each of 10 cm x 10 cm blots) and 500 cm² of membrane (5 each of 10 cm x 10 cm blots), respectively.

NOTES ON…Storage Conditions of Detector AP Chemiluminescent Blotting Kit

- The 5X Phosphatase Wash Solution Concentrate should be removed from the Detector AP Chemiluminescent Blotting Kit and stored at room temperature. All other components should be stored at 2-8°C.
- For optimal performance, Formamide Hybridization Buffer should be warmed (37-50°C) and aliquotted into DNase/RNase free tubes and then stored at 2-8°C. Do not store Formamide Hybridization Buffer at -20°C. Prolonged storage of this buffer at -20°C may result in decreased sensitivity.
- Reagents are stable for a minimum of one year when stored as instructed.

Required Supplies and Equipment Not Included

- Biotin-labeled nucleic acid probe
- Herring Sperm DNA (Catalog No. 60-00-14)
- 20X SSPE (See Appendix for preparation)
- SDS
- UV Transilluminator
- UV crosslinker or vacuum oven
- Capillary transfer apparatus
- Waterbath or hybridization oven
- Heat-sealed hybridization bags (Catalog No. 60-00-51) or hybridization bottles
- X-ray film and film cassettes or a chemiluminescent imaging system
- Positively charged nylon (Biodyne B, Catalog No. 60-00-50) or nitrocellulose membrane
- DEPC treated water
- 37% (v/v) Formaldehyde stock solution
- 10X MOPS Buffer (See Appendix for preparation)
- RNA sample loading buffer (See Appendix for preparation)
- Agarose
- Circulating horizontal electrophoresis tank
- 20X SSC (Catalog No. 50-86-05 or see Appendix for preparation)
- NaOH stock solution

Detector Northern Blotting At A Glance

Prehybridization
1 hour
↓
Hybridization
3-18 hours
↓
2X SSPE/0.5% SDS washes
2 X 15 min
↓
0.2X SSPE/0.5% SDS washes
2 X 30 min
↓
2X SSPE wash
5 min
↓
Detector Block
45 min
↓
AP-Streptavidin
30 min
↓
Phosphatase Wash Solution
3 x 5 min
↓
Phosphatase Assay Buffer
2 X 2 min
↓
CDP-Star
5 min
↓
Film Exposure
2 min - 2 hours

NOTES 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.
- 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 RNase Away (or something similar) or a 0.2N NaOH solution. Always wear gloves when working with RNA as human skin contains abundant amounts of RNases.

Gel Electrophoresis

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. When pouring the gel, however, the thickness should not exceed 6 mm.

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).	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.
2. Boil to dissolve the agarose. Cool to 60°C.	
3. In a fume hood, add 10 mL of 10X MOPS buffer to a final concentration of 1X. Swirl to mix.	
4. In a fume hood, add 4 mL of 37% Formaldehyde (12.3 M) to a final concentration of 0.45 M. Swirl to mix.	
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.	For an accurate time estimation for efficient transfer, it is important to maintain the 6 mm thickness of the gel. Do not allow the gel to solidify longer than 45 minutes.
6. Add RNA sample loading buffer to the RNA at a ratio of 2:1.	The RNA loading buffer contains formamide to reduce secondary structure in the RNA and ethidium bromide to visualize the RNA on the gel. See Appendix for instructions to prepare RNA loading buffer.
7. Denature the samples by incubating at 68°C for 10 min. Immediately place the samples on ice.	
8. Prepare running buffer: 100 mL 10X MOPS, 40 mL of 37% Formaldehyde, 860 mL DEPC Treated Water for a final concentration of 1X MOPS and 0.45 M Formaldehyde. Mix well.	
9. Fill the tank and pre-electrophorese the solidified gel at 50 V for 10 min.	
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.
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.
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 bands 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.

Akaline Transfer

Alkaline transfer is the preferred method for the transfer of RNA onto positively charged nylon membrane. This method is more reproducible than high salt overnight transfers, facilitating transfer within 3 hours regardless of the amount of RNA to be transferred.

Steps	Critical Points
1. Prepare 1 L of 5X SSC/10 mM 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. For Pall Biodyne B, prewet membrane in DEPC treated water followed by a 5 minutes wash in 5X SSC.
2. Treat the gel with 2 gel volumes of alkaline transfer buffer by washing 2 x 10 minutes at room temperature.	
3. Assemble a capillary transfer according to standard techniques using the transfer buffer as the solvent.	Always use clean forceps to handle membrane.
4. Transfer time will depend on the amount of RNA loaded on the gel. >3 µg of RNA, transfer 3 hours; 100 ng - 3 µg 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, neutralize and rinse the membrane for 5 min in a generous volume 5X SSC. Place membrane on filter paper 2-4 min 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. The 5X SSC wash is important to remove residual NaOH from the blot to prevent increased background.
6. Store membranes between two pieces of blotting paper and seal in a hybridization bag. Store bag in a cool and dry place until detection is performed.	See Hybridization and Detection Procedures, pages 37-38.

NOTES 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.

Prehybridization and Hybridization

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 0.1 mL/ cm^2 of membrane is recommended. Use the guidelines listed at the right.	Use 10 mL of Formamide Hybridization Buffer per 100 cm^2 membrane. (For example: a 10 cm X 10 cm membrane = 100 cm^2 = 10 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 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. For convenience, 10 mL of Hybridization Buffer regardless of blot size may be used. The hybridization buffer containing probe may be reused, and 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 100 µg per 1 mL of Formamide Hybridization Buffer that is used. If using KPL's Herring sperm DNA, add 5 µL per mL of 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.** Do not allow membranes to stick together or to the sides of the hybridization bag.
5. Incubate 1 hour with 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. Do not pipet less than 1 µL of probe. If probe is very concentrated, it may be diluted in small volume of a Hybridization Buffer for denaturation and added in a larger quantity to the hybridization.
7. Add the probe directly to the Prehybridization Buffer at 50 ng per mL of Buffer.	Pipet the probe directly into the buffer and not directly on the blot.
8. Incubate the membrane with gentle agitation 16-18 hours.	The desired hybridization temperature when using DNA probes is 42°C. Hybridization of RNA probes should be performed at 65°C - 68°C.
9. Prepare post-hybridization washes: 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 to 55°C if washing DNA probes or 65°C if washing RNA.	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. Equilibrate the 0.2X solution at the elevated temperature at least 2 hours prior to use.
10. Remove the membrane from the 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 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 the temperature equilibrated wash.	Generously cover the membrane with the wash solution. The elevated temperature wash should be temperature equilibrated at least 2 hours prior to use.
12. Perform the final wash in 2X 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

Steps	Critical Points
1. Incubate Northern blot with 1X Detector Block Solution for 45 minutes in a tray approximately the same size as the blot.	See Buffer Preparation Section for instructions on preparation of 1X Detector Block. Use at least 0.25 mL per cm^2. 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 AP-SA diluted 1/10,000.	Use at least 0.25 mL of diluted conjugate solution per cm^2.
3. Place membrane in a clean container. Wash the membrane in 1X Phosphatase Wash Solution 3 times for 5 minutes each.	Use at least 0.75 mL per cm^2.
4. Rinse the membrane 2 times for 2 minutes each in 1X 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. Use at least 0.4 mL per cm^2.
5. Incubate membrane for 5 minutes in CDP-Star Chemiluminescent Substrate. Blot membrane on filter paper to remove excess substrate. Place membrane in a hyb 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. (i.e. 10 cm x 10 cm = 100 cm^2 = 5 mL CDP-Star)
6. Develop film either manually or by using a mechanical processor.	

NOTES ON...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 to 4 hours persisting 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.

Stripping and Reprobing a Northern Blot

Membranes hybridized with biotinylated probes can be stripped and reprobed after detection with CDP-Star provided the membrane is never allowed to dry prior to stripping (i.e., store membrane in a covered container in 1X SSPE).

It is always easier to strip a Northern blot when using a DNA probe. RNA probes can be stripped, but it is more difficult to remove the probe in entirety. Any stripping protocol used with ^{32}P may be used with non-rad detection. Do not use alkaline methods for stripping Northern Blots. Similar results are often seen when using ^{32}P.

Method #1

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.

Method #2

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.

Buffer Preparation

Sufficient reagents are provided in the 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 in Northern blotting using this system, recommended preparation of kit buffers are detailed below. Required solutions not provided with the kit can be found in the Appendix, page 68.

Steps	Critical Points
1X Detector™ Block Solution– to be prepared fresh daily	
1. Based on the total desired 1X Detector Block volume, weigh out 0.2% w/v Detector Block Powder **for detection with AP and CDP-Star.**	If the block solution is not prepared daily, sensitivity could be reduced and background can 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. (Approximately 30 seconds to 1 minute)	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. Insure that all 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. Do not add the powder to the entire volume of the 1X Detection Block Solution. This will result in clumps that will be hard to solubilize.
3. Dilute the solution with 1:5 v/v 5X Detector Block Solution. Example, for 50 mL of 1X Detector Block: Detector Block Powder 0.1 g Molecular Biology Grade H_20 40 mL 5X Detector Block Solution 10 mL	

Steps	Critical Points
Phosphatase Wash Solution	
1. Dilute 1 part 5X 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 prior to use.
Phosphatase Assay Buffer	
1. Dilute 1 part 10X Assay Buffer with 9 parts molecular biology grade water. Mix well.	

In situ hybridization with Detector™ *In situ* Hybridization Kits

Pardue and Gall first described *in situ* hybridization for the localization of nucleic acids in tissues, chromosomes, and nuclei.[1] The method comprises three basic steps: fixation of a specimen (cells or tissue) on a microscope slide, hybridization of labeled probe to homologous fragments of genomic DNA, and detection of the tagged probe: target hybrids. Applications for this technique include detection of integration of exogenous DNA into the genome of cells, identification of chromosomal anomalies, cytogenetic analysis, and detection of viral infections.

Like nucleic acid blotting, detection may be facilitated via radioactivity, enzyme-reactive chromogenic substrate or fluorescent label. The latter two options offer advantages over radioisotopes as detailed throughout this guide. The method of choice depends upon the requirements of the assay.

KPL provides two systems for non-radioactive hybridization and detection of DNA *in situ*, one a colorimetric system and the other fluorescent (FISH). Both utilize a rapid and easy to use biotin-streptavidin system. A biotin-labeled nucleic acid probe is hybridized to target DNA on a microscope slide containing fixed cells or tissue. The biotin is recognized and binds with high affinity to the streptavidin conjugate. The streptavidin label is responsible for the subsequent visualization of the target DNA sequence. The DNADetector™ Chromogenic DNA *In situ* Hybridization Kit contains a streptavidin-horseradish peroxidase conjugate to react with a chromogenic substrate, TrueBlue, while the DNADetector Fluorescent *In situ* Hybridization Kit relies on a fluorescent label, CY™3, for detection.

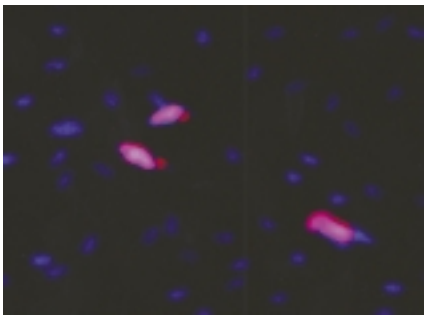
TrueBlue Peroxidase Substrate offers the greatest sensitivity over other chromogens like diaminobenzidine (DAB).[3] In addition, TrueBlue is preferred for superior resolution due to the very fine precipitate that is generated in the presence of HRP, unlike the large clumps of color yielded by BCIP/NBT. This blue precipitate is deposited at the site on the specimen where hybridization has occurred. Counterstaining by Orcein (for nuclear staining) and Eosin (for cytoplasmic staining) follows the substrate reaction to provide a red dye contrast to the blue signal.

For those assays that require even greater sensitivity, the DNADetector Fluorescent *in situ* Hybridization Kit was developed using a CY3-streptavidin conjugate. CY3 provides 5-10 times greater fluorescence than fluorescein-labeled probes and is more photostable than TRITC.[4] Bright fluorescence is observed 6 months after staining. No new filters are required; CY3 uses the same standard filters as TRITC. DAPI counterstain is included to provide contrast in staining.

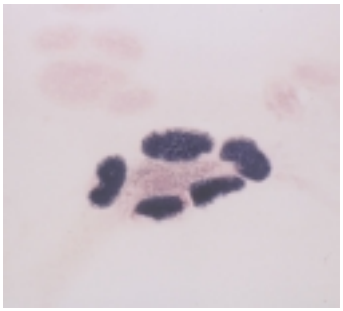
Due to the diversity of specific cells, tissues and probe hybridization conditions, it may be necessary to optimize tissue fixation and deproteinization, slide preparation and hybridization conditions for the specific sample. Recommendations for optimization of each of these steps are noted in the respective sections of the following protocols.

Assay Requirement Variables Impacting Outcome	
Sensitivity	• Accessibility of the target requires pre-treatment of tissue to expose site and block non-specific hybridization • Amount and type of label included in the probe • Method of detection
Resolution	• Varies from specific sites within a single cell • Probe label • Method of detection
Specificity	• Stringency of washes should be balanced with sensitivity requirements • Extent of similarity between the probe and sequences related to, but distinct from, the intended target
Sample type	• Fixation process should be optimized by tissue, cell sample
Number of genes/gene products to be detected simultaneously	• Availability of different gene-specific probes with different labels • Method(s) of detection multiplex of single method vs. combination of different methods (ex., colorimetric ISH and immunocytochemistry)
Convenience and safety	• Non-radioactive vs. isotopic probes - impacts stability, handling requirements and speed

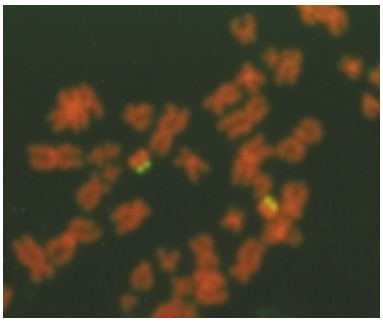
Table 1. Factors influencing selection of *in situ* hybridization and detection method.[2]



Cytomegalovirus infected cells detected with a biotinylated CMV probe and the DNADetector Fluorescent In situ Hybridization Kit using CY3-Streptavidin and DAPI.



Cytomegalovirus infected cells detected with a biotinylated CMV probe and the DNADetector Chromogenic In situ Hybridization Kit using HRP-Streptavidin, TrueBlue and Orcein



HT1080 tetraploid clone detected using a biotinylated pDP97 probe and the DNADetector Fluorescent In situ Hybridization Kit with CY2-Streptavidin and Propidium Iodide. Photo courtesy of E. Novotny, NHGRI/NIH.

Materials and Equipment

DNADetector Chromogenic *in situ* Hybridization Kit (Catalog No. 60-03-00 [60-03-00-A and 60-03-00-B]):

Kit Component (60-03-00-A)	Product Code	Volume	Storage Temperature
Hybridization Buffer (2X)	50-86-01	1 x 1.0 mL	2 - 8°C
Formamide	50-86-02	1 x 1.0 mL	2 - 8°C
DEPC-Treated H_2O	50-86-03	1 x 1.0 mL	2 - 8°C
Control Probe Hybridization Cocktail	60-00-08	1 x 50 µL	2 - 8°C
Streptavidin-HRP Conjugate	474-3002	1 x 0.1 mL	2 - 8°C
Conjugate Diluent Buffer	50-82-03	1 x 5.0 mL	2 - 8°C
Biotin Wash Solution Concentrate (10X)	50-63-09	3 x 200 mL	2 - 8°C
Control Slide	60-00-01	1 slide	-20°C

Kit Component (60-03-00-B)			
20X SSC	50-86-04	1 x 500 mL	Room Temperature
TrueBlue Peroxidase Substrate	71-00-68	1 x 50 mL	Room Temperature
Orcein (nuclear counterstain)	71-01-00	1 x 25 mL	Room Temperature
Eosin (10X) (cytoplasmic counterstain)	71-02-00	1 x 10 mL	Room Temperature

Materials and Equipment

DNADetector Fluorescent *in situ* Hybridization Kit (Catalog No. 60-05-00):

Kit Component	Product Code	Volume	Storage Temperature
Hybridization Buffer (2X)	50-86-01	1 x 1.0 mL	2 - 8°C
Formamide	50-86-02	1 x 1.0 mL	2 - 8°C
DEPC-Treated H_2O	50-86-03	1 x 1.0 mL	2 - 8°C
Control Probe Hybridization Cocktail	60-00-08	1 x 50 µL	2 - 8°C
CY3-Streptavidin	078-30-01	1 x 0.1 mg	2 - 8°C
Conjugate Diluent Buffer	50-82-03	1 x 5.0 mL	2 – 8°C
Wash Solution Concentrate (20X)	50-63-03	3 x 100 mL	2 - 8°C
DAPI	71-03-00	1 x 1.0 mg	2 - 8°C
Control Slide	60-00-01	1 slide	-20°C
20X SSC	50-86-04	1 x 500 mL	Room Temperature
Fluorescent Mounting Media	71-00-16	1 x 15 mL	Room Temperature

Each kit contains sufficient reagents for the hybridization and detection of 50 samples. The researcher supplies only the biotinylated probe specific for the target gene. Reagents are stable for a minimum of one year when stored as directed.

NOTES ON…Storage Conditions:

- Kit components require multiple storage temperatures. Please note the appropriate storage conditions for each component and place the components at the recommended temperature immediately upon receipt of the kit.
- The control slide provided in the kit may be stored at 2-8°C for up to 2 weeks, for long term stability it should be stored at -20°C.

Required Supplies and Equipment Not Included

- Glass Coplin jar, or equivalent, for high temperature incubations (above 37°C)
- Plastic or glass Coplin jars, or equivalent, for room temperature incubations
- Forceps
- Humidified chamber
- Water baths at 37°C and 60°C
- Incubator or heat block at 95°C
- Biotin-labeled nucleic acid probe
- Autoclaved 1.5 mL microcentrifuge tubes
- Autoclaved or sterile-filtered (0.2 µm) molecular biology grade water
- Treated microscope slides
- Rubber cement or coverslips and slide holders
- Organic mounting media, such as Permount (Fisher Scientific)
- Glass coverslips and parafilm
- Microscope/Fluorescent Microscope

Additional Supplies Needed for Tissue

- Xylene
- Reagent grade ethanol solutions (100%, 80%, 70%, and 50%)
- PBS
- Proteinase K

NOTES ON...Warnings and Precautions

- **Read ALL instructions thoroughly before using the kit.**
- Wear gloves when handling kit components and slides. Some components of the kit are sensitive to DNA degrading enzymes such as DNases.
- Some kit components contain hazardous materials (formamide, microcide, etc.). Handle these reagents using accepted laboratory safety procedures. Dispose of hazardous chemicals according to local, state and federal regulations.
- The streptavidin conjugate and counterstain are light sensitive. Keep caps closed and avoid prolonged exposure to light.
- For slide washes at temperatures above 37°C, the use of glass Coplin jars is recommended to achieve proper temperature equilibration of slides.
- Proper temperatures of heat blocks, water baths, and slide warmers are very important. It is necessary to achieve a temperature of at least 95°C for the specified time in order to denature the probe and target DNA.
- Keep all reagents at the designated temperatures stated in this manual, or on the labels, to maintain and prolong shelf life.
- Do not allow slides to dry out during the hybridization and detection procedures.

Experimental Design

Slide Preparation:

In order to ensure that specimens will remain attached to the slide during the *in situ* hybridization protocol, it is recommended that you pre-treat or "sub" your slides with an adhesive agent before mounting specimens. Reagents such as poly-L-lysine[5], 3-amino-propyltriethoxysilane (AES) and gelatin[6] have all been successfully used as subbing agents. The outline for AES treatment is as follows:

a. Incubate clean, dry glass slides in 2% AES (Aldrich) in acetone for 2 minutes.

b. Rinse slides 2 times in molecular biology grade H_2O and air dry.

c. Store slides at room temperature in a closed, dust-free container. Handle slides only by the corners.

Cell Preparation:

Cells can be applied to the slide using different techniques depending on the amount of sample available, the cell type being used, the applicability of your system to different slide growth chambers and the availability of cytospin centrifuges.

Use of chamber slides for growth of cells will allow the optimization of growth conditions to obtain a well spread and dense monolayer of cells. This may facilitate the ability to detect multiple *in situ* hybridization signals in a sample. Once optimal growth results are obtained, the medium may be removed and the cells then fixed on the slide.

Cells from cytological samples or cell cultures can be smeared on the slide to form a thin layer and then fixed on the slide. When only a small amount of cells are available it may be advantageous to concentrate the cells in a defined area of the slide using a cytospin centrifuge. Cells are then fixed on the slide.

Slide Fixation:

Optimal fixation conditions are critical for successful *in situ* hybridization. The reagents used for fixation, as well as the length of time a specimen is fixed, must be determined empirically for each sample type, as these parameters will influence morphological integrity and hybridization signal. There are two types of fixatives: precipitating fixatives and cross-linking fixatives. Precipitating fixatives, like ethanol or methanol/acetone, do not require further treatment with proteolytic enzymes. However, cells fixed by these methods are susceptible to loss of target nucleic acids. Cross-linking fixatives like formaldehyde or glutaraldehyde prevent loss of nucleic acids from their *in situ* origin, but subsequent proteolytic digestion is required in order to make the target nucleic acids accessible to probe. Further information on fixation may be found in references 7 – 9.

Paraffin-Embedded Tissue Sample Preparation:

Paraffin-embedded, formalin/paraformaldehyde fixed tissue sections of 4 - 6 micron thickness may be mounted on slides using the following method:

a. Float paraffin-embedded tissue sections in clean, distilled water.

b. Dip a silanized slide under the tissue section and scoop the section onto the slide by lifting the slide and attached tissue section out of the water.

c. Gently remove any bubbles that may have become trapped under the section with a soft brush.

d. Allow the sections to dry on the slides.

e. Bake the slides for 1 – 2 hours at 90°C or overnight at 65°C.

f. Store slides at room temperature in a closed container.

Deparaffinization and Rehydration of Cell or Tissue Sections:

Paraffin-embedded, mounted specimens must be deparaffinized, then pretreated with agents such as proteases, acids or detergents[10] to loosen the cross-links formed by fixatives and allow efficient access of the probe to the target. Incubate the slides sequentially in the series of solutions specified below. All incubations should be performed in Coplin jars. Use fresh reagents for each batch of slides (8 - 10 slides). Gently agitate the jars every few minutes during each incubation.

Step	Reagent	Time (minutes)
1	xylene	10
2	xylene	10
3	95% ethanol	5
4	95% ethanol	5
5	80% ethanol	5
6	70% ethanol	5
7	50% ethanol	5
8	molecular biology grade water	5

After the slides have been rehydrated, continue with protease digestion.

Proteinase K Digestion of Cells and Tissue Sections Fixed With Cross-linking Fixatives:

Optimal removal of proteins from specimens that have been fixed using cross-linking fixation methods is necessary to expose target nucleic acids for hybridization. The degree of deproteinization necessary must be determined empirically as it depends on the type of tissue used, the method of fixation, the thickness of the tissue section and whether the nucleic acid target is nuclear or cytoplasmic. Overdigestion may result in release of the target DNA from the tissue or loss of tissue morphology, while underdigestion prevents access of the probe to the target and will result in a weak hybridization signal. It is recommended that you titrate the amount of protease used and perform a time course to determine the optimal conditions for your samples.

Proteases used in in situ hybridization include Proteinase K, pronase, trypsin and 0.2N HCl. Proteinase K is the universal deproteinization agent. Prepare Proteinase K and digest specimen following manufacturer's recommendations.

Probe Preparation:

Use of these kits requires a biotin-labeled nucleic acid probe. Any of the methods described in Chapter 2 of this Technical Guide may be employed for this purpose. Additionally, fragments of DNA greater than 100 nucleotides in length may be labeled enzymatically by nick translation. For oligonucleotide probes, labeling can occur during the synthesis of the oligo by covalent attachment of a biotin or after synthesis by enzymatic end-labeling. DNA may also be labeled using a photoactivated biotin analog[11].

It is important that the probe generated is between 20-30 bases if it is an oligo, and 50-1000 base pairs in length if it is a genomic probe. The probe needs to be labeled in such a way that it has the optimal number of biotin groups to allow for greatest signal, yet not too many biotins that they interfere with hybridization or with the binding of streptavidin to the biotin. If high background is encountered, it may be due to the presence of the labeling components. G50 Microspin Purification Columns are not recommended to purify the labeled probe because biotin sticks to the resin resulting in loss of labeled probe. Instead, Spin-Pure filters (Catalog No. 60-00-53) are recommended to remove unincorporated nucleotides or primers with minimal loss of labeled product.

Hybridization:

Due to the unique melting temperatures and hybridization properties of different probes and target sequences, it will be necessary to optimize hybridization conditions for each probe and target specimen you use. The use of formamide lowers the melting temperature of the nucleic acid:nucleic acid hybrids, so hybridizations may be performed at lower temperatures. When the formamide concentration is increased and the hybridization temperature remains stable, the stringency of hybridization is increased. Formamide is supplied separately from the rest of the hybridization cocktail to allow for the optimization of hybridization conditions.

In addition to modifying formamide concentration, it may also be necessary to optimize the temperature at which you perform the hybridization reactions. Too low or nonstringent hybridization temperatures may result in high background due to non-specific hybridization or hybridization to shorter than desired stretches of homology.

Hybridization temperatures that are too high may result in loss of the ability of the probe to hybridize to the target at all. In addition, prolonged exposure of cells and tissue specimens to temperatures above 50°C may result in morphological alterations and structural abnormalities. Also, keep in mind that detection of single-copy genes is more difficult than detecting multi-copy genes. The hybridization conditions and probe concentration may need to be optimized depending upon the abundance of your target gene.

Recommended Controls:

Control for:	Determines:	Experiment:
Tissue Digestion	• Accessibility of the target to the probe. • Morphological integrity of specimen.	• Prepare a series of specimens at varying digestion times and concentrations of protease.
Probe Preparation	• Probe Specificity	• Use a positive control slide known to contain a specific target nucleic acid sequence. • Use a negative control slide known to lack a specific target nucleic acid sequence. • Hybridize a probe that is non-homologous with target nucleic acid (plasmid or genomic DNA of a different species). • Use no probe in hybridization cocktail. • Perform nuclease digestion of target nucleic acid. • Acetylate tissue with acetic anhydride to reduce the electrostatic binding of probe to positive charges on the tissue.
Detection	• Endogenous Biotin	• Omit hybridization step and incubate sample with SA conjugate, then detect.

NOTES ON...Assay Control

- A 2-well Control Slide and Control Hybridization Cocktail are included to facilitate familiarization with the methodology utilized in this kit. It will also serve as a test system for the type of results expected. It is recommended that this control assay be performed before attempting use of the kit with personal sample specimens.
- There should be no significant background staining. The infected cells in the (+) wells should be dark blue. If Orcein counterstain was used, nuclear material not bound by probe should appear light to dark pink. If Eosin counterstain was used, the cytoplasm should be stained light to dark red. The (-) well should appear pink to red due to the absence of probe in the hybridization cocktail.

DNADetector Chromogenic *In Situ* Hybridization At A Glance

DNADetector *in situ*
HYBRIDIZATION AND CHROMOGENIC DETECTION
AT A GLANCE
Total time: 3-4 hours for cells
+11–16 hours for tissue

Prehybridization
30 minutes
(no prehybridization of tissue)
↓
Hybridization
1-2 hours
(12–18 hours for tissue)
↓
2X SSC Washes
2 X 5 min
↓
HRP-Streptavidin Incubation
20 min
↓
Wash slides in Biotin Wash
3 X 5 min
↓
Detection
2-3 min
(5–10 minutes for tissue)
↓
Dry Slide
5 min.
↓
Counterstain
1-5 min.
↓
Air Dry
30 min.
↓
Dehydrate
↓
Mount Samples

Cell Hybridization:

Steps	Critical Points
1. Prepare cells as described in Experimental Design: Cell Preparation.	
2. Prewarm a humidified chamber or water bath to 37°C. Prewarm a water bath to 60°C.	
3. Prepare hybridization cocktail. Prepare sufficient reagent for both prehybridization and hybridization. Allow 10-50 µL per specimen per treatment.	See Buffer Preparation on page 52 for instructions on preparation of hybridization cocktail.
4. **Prehybridization:** Apply 10-50 µL hybridization cocktail to each sample. Save the remaining hybridization cocktail for addition of biotin-labeled probe (step 8).	
5. Incubate 30 minutes in a 37°C waterbath on top of a foam float or in a humidified chamber.	
6. Shake off hybridization cocktail from the slide and blot the slide with drying paper.	Be careful not to touch the specimen with the paper.
7. Prewarm an incubator or oven to 95°C.	
8. **Hybridization:** Add biotinylated probe to the remaining hybridization cocktail to a concentration of 1.0 ng/µL. Apply 10-50 µL hybridization cocktail containing probe to each specimen.	See Buffer Preparation on page 52 for instructions on preparation of probe hybridization cocktail.
9. Cover the slide with a coverslip. Alternatively the slide may be covered with a gasketed coverslip and placed in a slide holder.	
10. Place the slide at 95°C and incubate for 2 minutes to denature the target and probe DNA simultaneously.	
11. Transfer the slide to a 37°C humidified chamber and incubate 1-2 hours.	
12. Immediately after starting hybridization, prepare sufficient volume of 2X SSC for two incubations in Coplin jars. Prewarm 2X SSC to 60°C in one glass Coplin jar in a water bath. Place the remaining 2X SSC in a Coplin jar at room temperature.	See Buffer Preparation on page 52 for instructions on preparation of 2X SSC.
13. Following hybridization, remove the cover from the slide and place the slide in the Coplin jar containing prewarmed 2X SSC at 60°C for 5 minutes.	
14. Transfer the slide to the Coplin jar containing 2X SSC at room temperature and incubate for 5 minutes.	
15. Immediately proceed to chromogenic detection, page 49 or fluorescent detection, page 51.	

Tissue Hybridization

Steps	Critical Points
1. Prepare tissue sections as described in Experimental Design: Parrafin-Embedded Tissue Preparation.	
2. Prewarm a heat block or oven to 95°C. Prewarm a humidifed chamber or water bath to 37°C.	
3. Prepare sufficient hybridization cocktail for each sample. Allow about 10-50 µL per specimen per treatment.	See Buffer Preparation on page 52 for instructions on preparation of hybridization cocktail.
4. Add biotinylated probe to the hybridization cocktail to a concentration of 1.0 ng/µL. Apply 10-50 µL hybridization cocktail containing probe to each specimen.	No prehybridization is required.
5. Cover the slide with a coverslip and seal the slide around the edges with rubber cement.	Alternatively the slide may be covered with a gasketed HybCover and placed in a HybHolder.
6. Place the slide at 95°C and incubate for 10 minutes to denature the target and probe DNA simultaneously.	
7. Immediately transfer the slide to a 37°C humidified chamber and incubate 12-18 hours.	
8. Following incubation, prewarm a water bath to 60°C. Before proceeding to post-hybridization washes, make sufficient volume of 2X SSC for two incubations in Coplin jars. Prewarm 2X SSC to 60°C in one glass Coplin jar in a water bath. Place the remaining 2X SSC in a Coplin jar at room temperature.	See Buffer Preparation on page 52 for instructions on preparation of 2X SSC.
9. Remove the cover or sealed coverslip from the slide and place the slide in the prewarmed Coplin jar containing 2X SSC at 60°C for 5 minutes.	
10. Transfer the slide to the Coplin jar containing 2X SSC at room temperature and incubate for 5 minutes.	
11. Immediately proceed to chromogenic detection, page 49 or fluorescent detection, page 51.	

Chromogenic Detection:

Steps	Critical Points
1. Dilute the HRP-Streptavidin (amber vial, 474-3002) 1:100 in Conjugate Diluent Buffer (i.e. 1.0 µL HRP-SA + 99 µL Conjugate Diluent Buffer). Prepare sufficient volume for 20-50 µL per sample.	
2. Shake off the 2X SSC from the slide, and blot the slide with drying paper.	Do not allow the slide to dry. Be careful not to touch the specimen with the paper.
3. Immediately add 20-50 µL of the diluted HRP-Streptavidin to each sample. Cover the slide with a coverslip to prevent evaporation. Place slide in a 37°C humidified chamber for 20 minutes.	Alternatively the slide may be covered with a coverslip or piece of parafilm.
4. Prepare 1X Biotin Wash Solution. Dispense to 3 Coplin jars at room temperature.	See Buffer Preparation on page 52 for instructions on preparing 1X Biotin Wash.
5. Wash slides 5 minutes in each successive Coplin jar. Shake off Biotin Wash Solution from slide and blot dry.	
6. Apply TrueBlue to the slide.	Make sure that the edges of the specimen are covered.
7. Observe the development of color under the microscope to determine the optimal development time. Development should be optimal within 2-3 minutes for cells and 5-10 minutes for tissue at room temperature.	
8. When the signal has reached the desired intensity, stop color development by soaking the slide in a Coplin jar containing picopure water for 5-10 seconds.	
9. Air dry the slide for at least 5 minutes.	
10. Counterstain the sample as described below, using Orcein to stain nuclei, or Eosin to stain cytoplasm. (page 50)	

Counterstaining — Nuclear Counterstain:

Steps	Critical Points
1. Apply Orcein to the slide.	Make sure that the edges of the specimen are covered.
2. Incubate at room temperature for 3-5 minutes for cells and for 2 minutes for tissue.	
3. Rinse 5-10 seconds in a Coplin jar containing picopure water.	
4. Air dry slide for 30 minutes.	
5. Dehydrate slide and mount as described on page 50.	

Counterstaining — Cytoplasmic Counterstain:

Steps	Critical Points
1. Prepare enough 1X Eosin to cover the specimen (approximately 0.5 mL per slide). Mix well and store at room temperature.	See Buffer preparation on page 52 for instructions on preparing 1X Eosin.
2. Apply 1X Eosin to the slide.	Make sure that the edges of the specimen are covered.
3. Incubate at room temperature for 1-2 minutes for cells and 3 – 5 minutes for tissue.	
4. Rince 5 – 10 seconds in a Coplin Jar containing picopure water.	
5. Air dry slide for 30 minutes.	
6. Dehydrate slide and mount as described below.	

Dehydrate and Mount Slide:

Steps	Critical Points
1. Dip slide 5 times in a Coplin jar containing 95% EtOH. Transfer to a second Coplin jar containing 95% EtOH and dip 5 more times. Let the ethanol evaporate.	
2. Dip slide in 2 changes of xylene. Blot the excess xylene from the slide.	
3. Mount with an organic mounting media, such as Permount, and coverslip.	
4. Let the mounted slide dry overnight.	
5. View the slide under a microscope.	

DNADetector *in situ* HYBRIDIZATION AND FLUORESCENT DETECTION AT A GLANCE

Total time: 3-4 hours
(+11–16 hours for tissue)

Prehybridization
30 minutes
(no prehybridization of tissue)
↓
Hybridization
1-2 hours
(12–18 hours for tissue)
↓
2X SSC Washes
2 X 5 min
↓
CY3-Streptavidin Incubation
20 min
↓
Wash Slides
5 min
(3 x 5 min for tissue)
↓
Counterstain
3-5 min.
↓
Mount Samples

Fluorescent Detection Procedure:

While there are differences in the treatment of cells and tissues in preparation and hybridization of such samples, the procedures for detecting DNA in fixed cells and tissue sections are fundamentally the same.

Steps	Critical Points
1. Rehydrate lyophilized CY3-Streptavidin as instructed on page 53. Dilute the stock CY3 (red cap) streptavidin solution 1:100 in Conjugate Diluent Buffer (i.e. 1µL CY-SA + 99 µL Conjugate Diluent Buffer). Prepare 100 µL per sample.	
2. Shake off the 2X SSC from the slide, and blot the slide with drying paper.	Do not allow the slide to dry. Be careful not to touch the specimen with the paper.
3. Immediately add 20-50 µL of the diluted CY3-Streptavidin to each sample. Cover the slide with a coverslip to prevent evaporation. Place slide in a 37°C humidified chamber for 30 minutes.	Alternatively the slide may be covered with a HybCover or piece of parafilm.
4. Dispense 1X Wash Solution to 3 Coplin jars at room temperature.	See Buffer Preparation for instructions on preparing 1X Wash Solution.
5. Wash slides 5 minutes in each successive Coplin jar. Shake off Wash Solution from slide.	
6. Counterstain the sample with the appropriate fluorescent counterstain as described below.	

**For best results, do not allow slides to be exposed to light during detection procedure.*

Counterstaining:

Steps	Critical Points
1. Incubate specimen with approximately 50 µL of the DAPI counterstain for 3-5 minutes at room temperature in the dark (cover slide with aluminum foil).	Intense DAPI staining may mask other fluorescent signals. It is recommended that the concentration of DAPI be titrated to determine the optimal dilution.
2. Rinse 5-10 seconds in a Coplin jar containing PBS.	See page 53 on the Buffer Preperation Section for the Counterstain Rehydration.
3. Add a few drops Fluorescent Mounting Media and coverslip the slide.	
4. View slides with a fluorescent microscope using the appropriate excitation and barrier filters.	Suggested filters: CY3 552/565; DAPI 365/480 For dual color detection, use a triple-bandpass filter according to your microscope manufacturer.

Buffer Preparation:

In Situ Solution Preparation

Steps	Critical Points
2X SSC	
1. 1 part 20X SSC 9 parts autoclaved or sterile-filtered molecular biology grade water	2X SSC is to be prepared from a concentrated stock solution. See the Solution Preparation Section for the 20X SSC formulation.
Hybridization Cocktail	
1. 1 part Formamide (50-86-02) 1 part 2X Hybridization Buffer (50-86-01)	10 – 50 µL is typically sufficient for each sample. 50% Formamide is recommended by KPL, however, the stringency of hybridization can be decreased by decreasing the Formamide concentration. 10-20% Formamide can be prepared by decreasing the amount of formamide added and adjusting the volume with DEPC treated water. i.e. 10 µL Formamide, 40 µL DEPC treated water, and 50 µL of 2X Hybridization Buffer.
Probe Hybridization Cocktail	
1. Dilute the quantitated biotinylated probe directly into 10 – 50 µL of Hybridization Cocktail (prepared above) to a final concentration of 1 ng/µL.	When adding the probe, the volume must be no greater than 5% of the final volume of the cocktail. If your probe is too dilute, concentrate it in a speed vac or lyophilizer and resuspend it in the Hybridization cocktail. Hybridization conditions and probe concentrations may need to be optimized depending on the abundance of the target and the probe relative concentration.
CY3-Streptavidin Conjugate (Fluorescent Kit only)	
1. Rehydrate the lyophilized conjugate with 0.5 mL of Fluorescent Mounting Media (71-00-16) and 0.5 mL of autoclaved or sterile filtered molecular biology grade water. Mix well.	
2. Store this 0.1 mg/mL solution at –20C for 6 months.	
DAPI Counterstain (Fluorescent Kit only)	
1. Rehydrate the DAPI powder with 1.0 mL of autoclaved or sterile-filtered molecular biology grade water. Store this 1.0 mg/mL stock solution at –20ºC for 6 months.	
Humidified Chamber – for a dry incubator	
1. Line the bottom of a small box with a stack of paper towels soaked in water.	It is important to incubate the slides in a humidified chamber to prevent evaporation of reagents and dehydration of the specimen.
2. Place two pipets or rods in parallel on the towels (to place the slides on).	
3. Close the lid and incubate at the appropriate temperature to equilibrate.	
Humidified Chamber –for a waterbath with a lid	
1. Place a foam block in a tray inside the water bath. Place slides directly on the foam.	
1X EOSIN (Chromogenic Kit only)	
1. Dilute 1 part 10X Eosin into 9 parts autoclaved or sterile filtered molecular biology grade water. Mix well.	

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Troubleshooting Guide for Labeling Probes

This Troubleshooting chapter is designed to offer suggestions for overcoming potential issues that may occur during the non-radioactive labeling and detection of nucleic acids using Detector products. For additional technical support, contact KPL Technical Services at 800-638-3167 or 301-948-7755 or by e-mail at reagents@kpl.com.

Problem 1 - Probe does not quantitate or yield is low (<25 ng/µL)

Universal Issues

Possible Cause:	Tubes, reagents, template, or tips are contaminated.
Corrective Measure:	Use only clean, sterile or at least Dnase/Rnase free tips, tubes for each pipetting operation.
Possible Cause:	The polymerases are no longer functioning to their full potential.
Corrective Measure:	Enzymes are heat labile and must be stored in a non-frost free freezer. Test the components with the control template to identify compromised product.
Possible Cause:	The size of the probe is not appropriate for the type of labeling reaction.
Corrective Measure:	Use the appropriate biotinylation kit for your size needs: Random Primer Kit 300 bp – 1000 bp PCR Kit 100 bp – 1100 bp *In vitro* Transcription 80 b – 800 b
Possible Cause:	A component of the reaction is missing.
Corrective Measure:	Use the Template DNA included in the kit as a positive control for the components of the kit.

Random Priming

Possible Cause:	The template is not pure.
Corrective Measure:	Repurify your template. Make sure the nucleic acid template is purified very well. Kinases, nucleases, salts, ethanol, and protein contaminants will prevent the labeling reaction from occurring. If the labeling efficiency of your probe is unsatisfactory, use the Control Template provided with your kit as a measure of template purity. Probe yield from inadequately purified template will be much less than probe yield from control DNA.
Possible Cause:	The template is not pure.
Corrective Measure:	If the template is not grossly contaminated, it may be overcome by the addition of 2-4 µl of Klenow polymerase or increasing the incubation time to overnight.
Possible Cause:	The template is not denatured.
Corrective Measure:	Make sure to completely denature the double stranded template by heating to 95ºC for at least 5 minutes. The DNA must be single stranded to label.

PCR Labeling

Possible Cause:	Too few cycles were performed or extension time is too short.
Corrective Measure:	25-35 cycles is recommended and increasing the extension time may result in higher yield.
Possible Cause:	Annealing temperature is too high.
Corrective Measure:	Primer annealing temperature must be optimized empirically. Decrease in 2–4ºC increments.
Possible Cause:	Insufficient template has resulted in the inability of the primers to anneal to their complimentary sequences.
Corrective Measure:	Increase the concentration of the template.

PCR Labeling

Possible Cause:	Primers may not be optimally designed.
Corrective Measure:	Design primers that are 22-30 bp in length and a GC content of 45-60%.
Possible Cause:	The target template is difficult to amplify.
Corrective Measure:	The addition of 2-Betaine or DMSO may aid in the amplification of sequences with unusually high GC content and or secondary structure.
Possible Cause:	$MgCl_2$ concentration is too low.
Corrective Measure:	The optimal Mg^2+ concentration varies from 0.5 mM to 5 mM. Make sure you are using the correct amount for the your particular Taq polymerase. Make sure the $MgCl_2$ is completely thawed and thoroughly vortexed before use.

***In vitro* Transcription**

Possible Cause:	The DTT is old.
Corrective Measure:	Prepare and use fresh DTT.
Possible Cause:	The transcription buffer was not warmed to room temperature prior to use.
Corrective Measure:	The spermidine in the transcription buffer can cause the DNA to precipitate if it is used cold resulting in a low yield of transcript. Make sure to bring the transcription buffer to room temperature prior to use.
Possible Cause:	The template is not pure.
Corrective Measure:	Repurify your template. Make sure the nucleic acid template is purified very well. Kinases, nucleases, salts, ethanol, and protein contaminants will prevent the labeling reaction from occurring. If the labeling efficiency of your probe is unsatisfactory, use the Control Template provided with your kit as a measure of template purity. Probe yield from inadequately purified template will be much less than probe yield from control DNA.

Problem 2 - Multiple products and/or product is smeared (PCR Labeling Kit only)

Possible Cause:	Too many cycles are performed.
Corrective Measure:	Reduce the number of cycles to eliminate non-specific bands.
Possible Cause:	Annealing temperature too low.
Corrective Measure:	Increase the annealing and or extension temperature in 2-3ºC increments.
Possible Cause:	The primers may not be optimally designed.
Corrective Measure:	Design primers that are 22-30 bp in length and a GC content of 45-60%.
Possible Cause:	Too much enzyme in the mixture.
Corrective Measure:	0.5 to 2.5 units is suitable for most applications.
Possible Cause:	Template concentration is too high.
Corrective Measure:	Too much template can lead to an increase in mispriming events. Reduce the concentration of template in the reaction.

Problem 3 - Incomplete Transcript (*In vitro* Transcription Kit only)

Possible Cause:	A terminator sequence for SP6 or T7 polymerase lies within the DNA template.
Corrective Measure:	Subclone the template behind a different RNA polymerase promoter.
Possible Cause:	Low incorporation of the nucleotides.
Corrective Measure:	Lowering the incubation temperature to room temperature may increase the amount of full length transcripts.

Problem 4 - Longer transcripts than expected (*In vitro* Transcription Kit only)

Possible Cause:	The plasmid was not digested completely.
Corrective Measure:	Analyze digested and undigested DNA to make sure the digest is complete.
Possible Cause:	A restriction enzyme was used that leaves a 3′ overhang.
Corrective Measure:	Use an enzyme which leaves a 5′ overhang or a blunt end. The 3′ end may be filled in using Klenow polymerase.

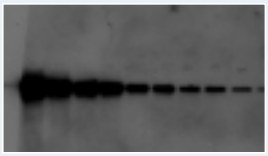
Troubleshooting Guide for Detection on Membranes

Problem 1 - High Background over the entire blot

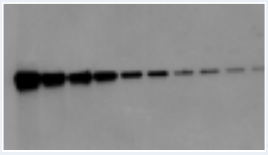
Universal Issues

Possible Cause:	Over-exposed film.
Corrective Measure:	Shorten the exposure time to film.

A



B

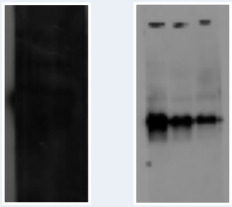


C

Detection of a Southern blot using the AP Chemiluminescent Kit. Detection of a 2-fold dilution series starting with 50 pg of target, membrane was exposed to film for 10 minutes (A), 2 minutes (B), and 30 seconds (C).

Possible Cause:	Excess probe in hybridization buffer.
Corrective Measure:	Quantitate the probe and add only 50 ng per mL of hybridization buffer i.e. 500 ng in 10 mL of hybridization buffer.
Possible Cause:	Membrane dried out at some point during the assay procedure.
Corrective Measure:	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.
Possible Cause:	Insufficient blocking.
Corrective Measure:	Make sure the Detector Block Powder is completely in solution. There should be no clumps of powder remaining in the solution.
Possible Cause:	Too much conjugate was added.
Corrective Measure:	Add the conjugate at 1:10,000 for AP i.e. 5 µl of conjugate to 50 mL of diluent or 1:500 for HRP i.e. 100 µl to 50 mL of diluent.

Possible Cause:	Post-hybridization washes were not stringent enough to wash the probe off of the membrane.
Corrective Measure:	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.



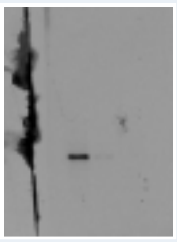
A B

Detection of a 18s rNA on Northern blots. Image A represents detection where with the post-hybridization buffer was not temperature equilibrated prior to the wash and Image B is detected per the standard protocol.

Problem 2 - Spotty Background, not all over the blot

Universal Issues

Possible Cause:	Particulate in solutions or dusty containers used.
Corrective Measure:	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.
Possible Cause:	Substrate has come into contact with the X-ray film.
Corrective Measure:	Seal the membrane in a plastic sheet protector or hybridization bag prior to exposure to film.



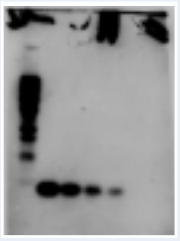
Background on the side of the image is caused from the substrate coming in contact with the film.

Problem 3 - Smudges or spots on film

Universal Issues

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 before handling the membrane if the forceps have been in the conjugate solution.



Detection of a Southern blot using the HRP Chemiluminescent Kit. Forceps contaminated with conjugate were used to handle membrane prior to the addition of substrate.

Problem 4 - Signal appearing as scratches on film

Universal Issues

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. When cutting the membrane do not use razor blades or scissors that have rust build up on them.

Possible Cause:
Corrective Measure:

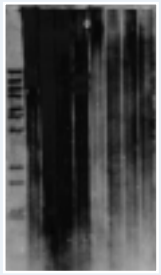
Static electricity was exposed to the film.
Do not wear gloves when handling the film because it can produce static electricity which can cause the appearance of lightning bolts when developed.

Detection of a dot blot using the HRP Chemiluminescent Kit showing the effect of static electricity that has exposed the film.

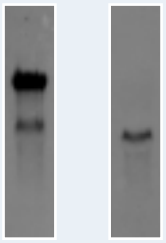
Problem 5 - Lane specific background or smears within gel lanes

Universal Issues

Possible Cause:	Nucleic acid has degraded.
Corrective Measure:	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.
Possible Cause:	Non-specific hybridization.
Corrective Measure:	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 55ºC – 60ºC for DNA probes or 68ºC for RNA probes.



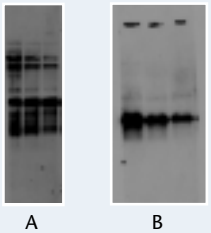
Southern blot demonstrating lane specific background due to low stringent washes. Detection of various digests of human genomic DNA with biotinylated HPV probes using the HRP Southern Chemiluminescent Kit.



A B

Detection of c-myc on Northern blots using the standard recommended conditions – note the detection of nonspecific signal from the 28s rRNA (A). Detection of c-myc after modifying the stringency by increasing the hybridization temperature and the post-hybridization washes from 65ºC – 68ºC to eliminate nonspecific signal (B).

Possible Cause:	Non-specific hybridization.
Corrective Measure:	Make sure to include sheared, denatured herring sperm DNA in the hybridization cocktail.
Possible Cause:	Probe concentration too high.
Corrective Measure:	Decrease the probe concentration in the hybridization cocktail.



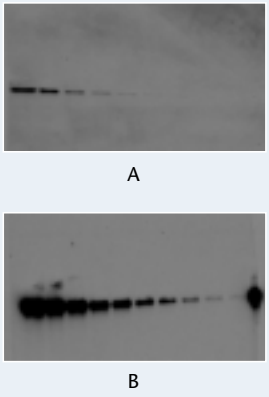
A B

Detection of a Northern blot with too much 18s RNA probe added to the hybridization buffer (A) and the correct amount added (B). Both blots were detected with the same stringent wash conditions.

Problem 6 - Low Signal

Universal Issues

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.
Possible Cause: **Corrective Measure:**	Inefficient transfer Verify the transfer of nucleic acids by viewing the gel and membrane under UV illumination to check that the nucleic acid has transferred from the gel to the membrane. 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 too long under alkaline conditions could cause "blow through" in which the nucleic acids could transfer through the membrane. Follow the general guidelines in this manual.
Possible Cause: **Corrective Measure:**	Too much powder was added to the 1X Detector Block Solution. Make sure to add the correct amount w/v for the appropriate enzyme. Use 0.2% for AP and 1% for HRP.



A

B

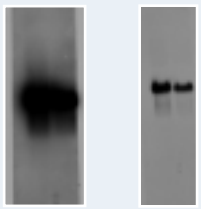
Detection of Southern blots using the AP Chemiluminescent Kit with 1% powder added to the Block (A) and 0.2% added (B). Too much powder decreases signal.

Possible Cause: **Corrective Measure:**	Hybridization conditions too stringent. Decrease the temperature of hybridization.
Possible Cause: **Corrective Measure:**	Post-hybridization wash conditions are too stringent. Decrease the wash stringency by increasing the salt concentration and/or decreasing the temperature of the washes.
Possible Cause: **Corrective Measure:**	Insufficient biotinylated probe added to the hybridization. Make sure to quantitate your probe using the Quantitation Standard. 50 ng per mL of Hybridization Cocktail should be added. If the background is low, 100 ng per mL may be added.
Possible Cause: **Corrective Measure:**	Degradation of probe. Check the integrity of the probe by running it on an agarose gel. RNA and PCR probes should be a single size. The random primed probes will appear as a smear with the majority of the probe ~200-300 bp.

Problem 7 - Diffuse Signal

Universal Issues

Possible Cause: Excessive space between the membrane and the film.
Corrective Measure: 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.



A B

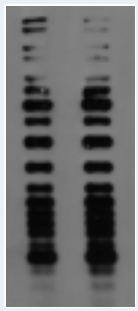
Films depicting a cassette that was not completely closed during the membrane exposure to film (A) and the same blot exposed to film with a tight closure of the cassette (B).

Possible Cause: The DNA side of the membrane faces away from the film.
Corrective Measure: 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

Universal Issues

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



Detection of a blot of the GeneRuler bioinylated ladder. Note the circular pattern of weak signal due to the presence of an air bubble during transfer.

Problem 9 - Ghost images i.e. faint signal development next to actual bands

Universal Issues

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

Troubleshooting *In Situ* Hybridization and Detection

Problem 1 - Low or No Signal

Universal Issues

Possible Cause:	The amount of the fluorescent counterstain used is too high (fluorescent kit only).
Corrective Measure:	Titrate down the amount of counterstain used. If it is too concentrated, it will mask the fluorescence of the specific signal.
Possible Cause:	Target and probe were not completely denatured.
Corrective Measure:	Check temperature of heating apparatus; adjust temperature accordingly or increase the heating time.
Possible Cause:	Tissue or cells were over-fixed.
Corrective Measure:	Decrease the length of the fixation step or change to an alternate fixation methodology.
Possible Cause:	Tissue was under-digested.
Corrective Measure:	Increase temperature, time or concentration of protease in the digestion step.
Possible Cause:	Tissue was over-digested resulting in the loss of target specimen from the slide or poor morphology.
Corrective Measure:	Decrease temperature, time or concentration of protease in the digestion step.
Possible Cause:	Not enough probe in the hybridization reaction.
Corrective Measure:	Increase the probe concentration.
Possible Cause:	Hybridization conditions were too stringent.
Corrective Measure:	Decrease the temperature of hybridization.
Possible Cause:	Hybridization conditions were too stringent.
Corrective Measure:	Decrease the concentration of the formamide in the hybridization cocktail.
Possible Cause:	Post-hybridization wash was too stringent.
Corrective Measure:	Decrease the temperature or time of the post-hybridization wash.
Possible Cause:	Labeling of the probe with biotin was inefficient.
Corrective Measure:	See the troubleshooting guide for the labeling kits.
Possible Cause:	Slide was pretreated improperly.
Corrective Measure:	Use a different subbing agent such as AES.

Problem 2 - High background or nonspecific signal

Universal Issues

Possible Cause:	Probe hybridizes to non-homologous DNA.
Corrective Measure:	Test the probe in a Southern blot to confirm the homology to the DNA.
Possible Cause:	Probe was not purified from unincorporated nucleotides prior to use.
Corrective Measure:	Purify probes with a SpinPure filter or a gel filtration column specifically designed for purification of biotinylated DNA.
Possible Cause:	The post-hybridization washes were not stringent enough.
Corrective Measure:	Increase the temperature or decrease the salt concentration of the washes to reduce non-specific hybridization.
Possible Cause:	Too much probe was used
Corrective Measure:	Make sure to quantitate the probe and use the recommended amount. If background persists, reduce the probe concentration in the hybridization.
Possible Cause:	The hybridization conditions were not stringent enough.
Corrective Measure:	Increase the temperature of the hybridization step or increase the concentration of the Formamide in the hybridization cocktail.
Possible Cause:	Sample was allowed to dry during detection.
Corrective Measure:	Keep sample moving through all of the detection steps quickly. Do not let the sample dry out.
Possible Cause:	Endogenous biotin is present in the sample causing non-specific detection.
Corrective Measure:	Include a control sample in the detection with no probe to identify if this is the problem. If so, block the specimen with free streptavidin and then saturate the streptavidin with biotin. Wash the sample well after blocking.

Miscellaneous Applications

Colony Lifts using Detector HRP Chemiluminescent Blotting Kits

Note: For best results, KPL recommends using a 1.2 micron pore size nylon membrane.

1. Plate cells on selective media (i.e. LB + amp, 50 µg/mL) and incubate inverted overnight at 37°C.
2. Pre-cool plates at 4°C for 30 minutes. Carefully lay membrane onto the agar plate containing the colonies. Beginning at one edge of the plate lay the membrane down smoothly, avoiding bubbles.
3. Mark membrane with India ink in a distinctive asymmetrical pattern.
4. Remove membrane from the plate after 1 minute. A second lift may be performed on the same plate by laying down a second membrane for 1 minute. 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. Place membrane onto filter paper saturated with 2X SSC for 5 minutes.
10. UV crosslink DNA to the membrane according to manufacturer's instructions. Alternatively, the membrane can be baked for 30 minutes at 80°C.
11. Immediately continue with the Hybridization.

Hybridization

1. Prehybridization: Place the Hybridization Buffer at 37°C and heat until solution is homogeneous.
2. Prepare prehybridization solution by adding Herring Sperm DNA (Catalog No. 60-00-14) to Hybridization Buffer to a final concentration of 200 µg/mL. Prepare at least 0.05 mL Hybridization Buffer per cm^2 membrane.
3. Place membranes in a hybridization bag or bottle and add 0.05 mL prehybridization solution per cm^2 membrane. Incubate 30 minutes with constant agitation at 42°C.
4. Hybridization: Heat biotinylated probe to 95°C for 10 minutes and place it on ice immediately. Add denatured probe directly to the prehybridization solution used in step 2 to a final concentration of 50 ng/mL. Be careful to pipet the probe into the solution and not directly onto the blot.
5. Incubate membranes at 42°C for 3-16 hours with agitation.
6. Following hybridization, remove membranes from the hybridization buffer. Hybridization buffer may be stored at 4°C and reused. Note: To reuse hybridization solution, heat to 68°C for 10 minutes to denature probe.
7. Wash membranes in 0.5X SSPE for 15 minutes at room temperature. Equilibrate a second aliquot of 0.5X SSPE to the hybridization temperature. Perform a second wash in temperature equilibrated 0.5X SSPE for 15 minutes. Washes may be modified to contain different concentrations of SSC, SSPE, and/or SDS to control stringency of the probe-target hybrid, however, the final wash must not contain any SDS. Residual SDS carried over onto the membrane during the blocking step may result in high background.

Detection

Chemiluminescent Detection:

Perform all steps at room temperature with gentle agitation. All working volumes listed may be increased. Stand alone solutions are available to meet increased volume requirements.

1. Based on the total desired 1X Detector Block volume, weigh out 1% 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 can 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. (Approximately 30 seconds to 1 minute) 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. Insure that all 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. Do not add the powder to the entire volume of the 1X Detection Block Solution. This will result in clumps that will be hard to solubilize.
3. Dilute the solution with 1:5 v/v 5X Detector Block Solution. For example, for 50 mL of 1X Detector Block use 0.5 g of Detector Block Powder; 40 mL of Molecular Biology Grade H_20; and 10 mL of 5X Detector Block Solution.
4. Place membranes in a heat-sealed hybridization bag or a tray approximately the same size as the membranes. Add at least 0.2 mL diluted Detector Block per cm^2 membrane and incubate for 30 minutes at room temperature.
5. Pour blocking solution off the membranes. Dilute HRP-labeled Streptavidin 1:500 in fresh Detector Block Solution. Mix well and pour the conjugate/blocking solution on the membranes. Incubate membranes for 20 minutes at room temperature.

6. Dilute Biotin Wash Solution Concentrate 1:10 in molecular biology grade water. Transfer membranes to a clean container and wash 3 times for 5 minutes each using at least 0.4 mL diluted wash solution per cm^2 membrane.
7. Prepare enough LumiGLO Chemiluminescent Substrate to completely immerse the membrane by mixing equal volumes of Solutions A and B. Incubate membrane one minute in the LumiGLO working solution.
8. Remove membranes from LumiGLO and touch a clean piece of filter paper to absorb excess solution. Place membranes in a hybridization bag or between plastic sheet protectors or transparency films. Smooth out bubbles, and place the plastic-covered membranes in an X-ray film cassette. Make sure no substrate has leaked out from between the plastic covering onto the film.
9. Expose membrane to X-ray film (DNA-side facing the film) at room temperature. KPL recommends an initial exposure of 1-2 minutes for colony lifts. The signal obtained from the first exposure allows the researcher to determine an exposure time for optimal signal.

Note: 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.

10. Develop film either manually or using a mechanical processor.

Timp-2 plasmid transformed into E. coli. Detected using a biotinylated timp-2 probe, HRP-Streptavidin and LumiGLO: 1 minute film exposure.

Colorimetric Detection with TMB:

Following detection steps 1 - 5, the membrane may also be detected chromogenically using TMB Membrane Substrate (Catalog No. 50-77-18).

1. Immerse membranes in TMB Membrane Substrate until desired endpoint is seen (2-10 minutes).
2. Rinse membranes in molecular biology grade water for 1 minute to stop the reaction, then air dry.
3. Store blots in the dark to avoid rapid fading.

Visit KPL online at www.kpl.com to learn more about kits and reagents for the sensitive non-radioactive detection of nucleic acids and proteins.

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 nylon membrane onto the surface of the plate (KPL recommends 1.2 µm Biodyne A from Pall Gelman Sciences). 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.

Note: Filter paper that is too wet will result in diffusion of the phage. Use just enough solution to evenly wet the filter paper.

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 min.
9. Fix DNA to the membrane by baking at 80°C for 30 minutes, or by UV crosslinking.

Prehybridization:

Use at least 0.06 mL Formamide Hybridization Buffer per cm^2 membrane. (For example: 10 cm x 10 cm membrane = 100 cm^2; 0.06 mL x 100 = 6 ml of Hybridization Buffer required.) The volume of buffer may be adjusted depending on the size of the membrane and the vessel used for hybridization.

a. Prepare prehybridization solution by adding sheared and denatured herring sperm DNA (Catalog No. 60-00-14) to the Formamide Hybridization Buffer to a final concentration of 200 µg/mL. If using non-denatured blocking DNA, heat denature for 5 minutes, cool quickly on ice and add to prehybridization solution.

b. Place the membrane in a hybridization bag or bottle and add at least 6.0 mL prehybridization solution per 100 cm^2 membrane. Incubate 1 hour with constant agitation at 42°C.

Hybridization:

a. Heat the probe to 95°C for 10 minutes and place it on ice immediately.

b. Add the biotinylated probe directly into prehybridization solution to a final concentration of 50 ng/mL. Be careful to pipette the probe into the solution and not directly onto the blot.

c. Incubate membrane 3 - 16 hours at 42°C with agitation.

Post Hybridization Washes:

a. Following hybridization, remove membrane from the hybridization buffer. Store the hybridization buffer in a sterile conical polypropylene tube at 2-8°C for reuse.

b. Wash membrane 2 x 15 minutes in a generous volume (at least 1 mL per cm^2 of membrane) of 2 X SSPE/0.1% SDS at room temperature.

c. Equilibrate a second wash of 0.2X SSPE/0.1% SDS to 55°C. Wash 2 x 15 minutes in temperature equilibrated wash at 55°C.

d. Perform a final wash for 5 minutes with 2X SSPE at room temperature to remove residual SDS.

Note: Washes may be modified to contain different concentrations of SSC, SSPE, and/or SDS to control stringency of the probe-target hybrid. It is recommended that this be optimized for each individual probe.

Detection:

Perform all steps at room temperature with gentle agitation.

1. **Prepare enough 1X blocking/diluent solution for the blocking step and for the HRP-SA conjugate dilution.** Prepare at least 0.3 mL 1X blocking/diluent solution per cm^2 membrane (i.e. 10 cm x 10 cm membrane = 100 cm^2; 0.3 mL x 100 = 30 mL of 1X blocking/diluent solution). Based on the total desired 1X Detector Block volume, weigh out 1% 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 can increase.
2. Place membrane in a heat sealed hybridization bag or a tray approximately the same size as the membrane, incubate with 1X blocking/diluent solution (from step 1) for 30 minutes. Use at least 0.3 mL 1X blocking solution per cm^2 membrane (i.e. 10 cm x 10 cm membrane = 100 cm^2; 0.3 mL x 100 = 30 ml of block solution).
3. a. Prepare HRP-SA at 1:500 in fresh Detection Block (60 µl + 30 mL blocking solution).

   b. Incubate membrane for 20 minutes at room temperature in the conjugate solution.
4. Dilute Biotin Wash Solution Concentrate 1:10 in molecular biology grade water. Transfer membranes to a clean container and wash 3 times for 5 minutes each using at least 0.4 mL diluted wash solution per cm^2 membrane.
5. Prepare enough LumiGLO Chemiluminescent Substrate to completely immerse the membrane by mixing equal volumes of Solutions A and B. Incubate membrane one minute in the LumiGLO working solution.
6. Remove membranes from LumiGLO and touch a clean piece of filter paper to absorb excess solution. Place membranes in a hybridization bag or between plastic sheet protectors or transparency films. Smooth out bubbles, and place the plastic-covered membranes in an X-ray film cassette. Make sure no substrate has leaked out from between the plastic covering onto the film.
7. Expose membrane to X-ray film (DNA-side facing the film) at room temperature. KPL recommends an initial exposure of 1-2 minutes for colony lifts. The signal obtained from the first exposure allows the researcher to determine an exposure time for optimal signal.

Note: 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.

8. Develop film either manually or using a mechanical processor.

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. The range of detection which can be expected is 1 ng - 0.1 pg of DNA.

1. With a pencil 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 at least 10 minutes. UV-crosslink DNA to the damp membrane according to manufacturers instructions or bake for 30 minutes at 80°C.
7. Hybridization and detection of dot blots follow the same protocols and guidelines for the HRP Chemiluminescent and AP Chemiluminescent Detection Kits.
8. Once the dot blots are prepared, follow the hybridization and detection protocols on page 30 for HRP detection and page 28 for AP detection.

Additional Buffer Recipes

10X MOPS Buffer

1. Mix:
   0.2 M MOPS, pH 7.0
   0.05 M Sodium Acetate
   10 mM EDTA, pH 8.0
   In 1 liter of DEPC treated Water
2. Sterile filter solution.

RNA Sample Loading Buffer

1. Mix:
   62.5% Deionized formamide
   1.14 M Formaldehyde
   200 µg/mL Bromophenol blue
   200 µg/mL Xylene cyanole
   1.25X MOPS Buffer 50 µg/mL Ethidium Bromide

0.1 M DTT (Dithiothreitol)

1. Prepare a 1 M stock solution by diluting 0.154g of DTT in 1mL of DEPC treated water.
2. Dilute 0.1 mL of the 1 M solution in 0.9 mL of DEPC treated water. Use the 0.1 M solution in the protocol. For optimal performance, this solution must be prepared fresh each time.

20X SSC (Catalog No. 50-86-05)

1. Mix:
   3.0 M NaCl
   300 mM Sodium Citrate
2. pH to 7.5 with NaOH
3. Sterile filter or autoclave solution.

LB Media

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

NZY Media

1. Mix:
   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 solution.

10X TE (Tris-EDTA) Buffer

1. 100 mM Tris-HCl
   10 mM EDTA
2. pH to 8.0 with HCl
3. Sterile filter or autoclave solution.

20X SSPE

1. Mix:
   3.0 M NaCl
   0.2 M sodium phosphate (mono-basic)
   0.02 M EDTA
2. pH to 7.4 with NaOH
3. Sterile filter or autoclave solution.

Related Products:

Nucleic Acid Labeling Kits	Catalog No.	Size
Detector Random Primer DNA Biotinylation Kit	60-01-00	30 reactions
Detector PCR DNA Biotinylation Kit	60-01-01	30 reactions
Detector RNA in vitro Transcription Biotinylation Kit	60-01-02	20 reactions

Nucleic Acid Detection Kits	Catalog No.	Size
Detector AP Chemiluminescent Blotting Kit	54-30-01	2000 cm^2
Detector AP Chemiluminescent Blotting Kit	54-30-02	500 cm^2
Detector HRP Chemiluminescent Blotting Kit	54-30-00	2000 cm^2
DNADetector Chromogenic *in situ* Hybridization Kit	60-03-00	50 slides
DNADetector Fluorescent *in situ* Hybridization Kit	60-05-00	50 slides

Molecular Biology Support Reagents/Accessories	Catalog No.	Size
Biodyne B Nylon Membrane	60-00-50	20 cm x 1 m
Hybridization Bags	60-00-51	50 bags
Detector Block, 5X	71-83-00	240 mL
HRP-labeled Streptavidin	474-3000	1 mL
AP-labeled Streptavidin	475-3000	1 mL
Biotin Wash Solution Concentrate, 10X	50-63-06	200 mL
Spin-Pure Filters	60-00-53	5 filters
Membrane Hybridization Buffer	50-86-08	240 mL
Formamide Hybridization Buffer	50-86-10	240 mL
AP Wash Solution Concentrate, 5X	50-63-15	600 mL
AP Assay Buffer	50-63-14	200 mL
Fluorescent Mounting Media	71-00-16	15 mL
Herring Sperm DNA	60-00-14	40 mg
GeneRuler Biotinylated DNA Ladder	600-0008	20-50 lanes
20X SSC	50-86-05	1 L
Silver Enhancer Kit for Microscopy	50-22-01	50 mL
Wash Solution Concentrate Kit 20X	50-63-00	800 mL

AP Substrates	Catalog No.	Size
CDP-Star Chemiluminescent Substrate	50-60-05	100 mL
BCIP/NBT Phosphatase Substrate Systems	50-81-00	300 mL
BCIP/NBT 1-Component Phosphatase Substrate Kit	50-81-07	6 x 100 mL
BCIP/NBT 1-Component Substrate	50-81-10	1000 mL
BCIP/NBT 1-Component Substrate	50-81-18	100 mL

HRP Substrates	Catalog No.	Size
LumiGLO Chemiluminescent Substrate	54-61-00	240 mL
LumiGLO Chemiluminescent Substrate	54-61-01	720 mL
LumiGLO Chemiluminescent Substrate	54-61-02	60 mL
TMB Membrane Peroxidase Substrate System	50-77-00	440 mL
TMB 1-Component Membrane Peroxidase Substrate	50-77-03	200 mL
TMB 1-Component Membrane Peroxidase Substrate	50-77-04	1000 mL
TMB 1-Component Membrane Peroxidase Substrate	50-77-18	100 mL

Conjugates:	Catalog No.	Size
AP-labeled Streptavidin	475-3000	1 mL
HRP-labeled Streptavidin	474-3000	1 mL
FITC-labeled Streptavidin	072-30-00	1 mg
TRITC-labeled Streptavidin	073-30-00	1 mg
Texas Red-labeled Streptavidin	0717-30-00	1 mg
CY™2-labeled Streptavidin	077-30-00	1 mg
CY™3-labeled Streptavidin	078-30-00	1 mg
CY™5-labeled Streptavidin	079-30-00	1 mg
Gold-labeled Goat anti-Human IgG (H+L) System	57-10-06	40 nm
Gold-labeled Goat anti-Rabbit IgG (H+L) System	57-15-06	40 nm
Gold-labeled Goat anti-Mouse IgG (H+L) System	57-18-06	40 nm
Gold-labeled Streptavidin System	57-30-06	40 nm
Gold-labeled Goat anti-Biotin System	57-40-06	40 nm

Stains & Counterstains:	Catalog No.	Size
TrueBlue Peroxidase Substrate	71-00-67	10 mL
TrueBlue Peroxidase Substrate	71-00-64	50 mL
TrueBlue Peroxidase Substrate	50-78-02	200 mL
Orcein- nuclear counterstain	71-01-01	50 mL
Eosin Y – cytoplasmic counterstain	71-02-01	10 mL
DAPI – fluorescent counterstatin	71-03-01	1 mg
Propidium Iodide – fluorescent counterstain	71-04-01	1 mg

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