DIG Application Manual

for Filter Hybridization

Intended use

Our preparations are exclusively intended to be used in life science research applications.* They must not be used in or on human beings since they were neither tested nor intended for such utilization.

Preparations with hazardous substances

Our preparations may represent hazardous substances to work with. The dangers which, to our knowledge, are involved in the handling of these preparations (*e.g.*, harmful, irritant, toxic, etc.), are separately mentioned on the labels of the packages or on the pack inserts; if for certain preparations such danger references are missing, this should not lead to the conclusion that the corresponding preparation is harmless. All preparations should only be handled by trained personnel.

Preparations of human origin

The material has been prepared exclusively from blood that was tested for Hbs antigen and for the presence of antibodies to the HIV-1, HIV-2, HCV and found to be negative. Nevertheless, since no testing method can offer complete assurance regarding the absence of infectious agents, products of human origin should be handled in a manner as recommended for any potentially infectious human serum or blood specimen.

Liability

The user is responsible for the correct handling of the products and must follow the instructions of the pack insert and warnings on the label.

Roche Diagnostics shall not assume any liability for damages resulting from wrong handling of such products.

Because of its outstanding tactile sensitivity, the Red-Eyed-Tree Frog (*Agalychnis callidryas*) was chosen to represent the high sensitivity of the DIG System.

* exeption: instruments specifically intended for *in-vitro* diagnostic use.

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DIG Application Manual

for Filter Hybridization

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Introduction

1

Introduction to the DIG System and the *DIG Application Manual*

The DIG System is a simple, effective system for nonradioactive labeling and detection of nucleic acids. Compared to radioactive labeling and detection techniques, the DIG System offers many advantages, including:

- The labeling and detection technology is safe.
- Labeled probes are stable and can be stored for at least a year.
- Hybridization solutions can be reused several times.

In fact, with the DIG System, you can have all the advantages of radioactive hybridization assays (*e.g.* familiar labeling and hybridization techniques, high sensitivity, low background), with none of the disadvantages (*e.g.* hazardous materials, difficult waste disposal, long assay times, instability of labeled probe). This combination of features makes the DIG System ideal for labs that perform many hybridization experiments ("high throughput" labs).

An Overview of the DIG Nonradioactive Nucleic Acid Labeling and Detection System

The flexibility and power of the DIG System allows you to:

- *Choose the best hybridization probe for your application.*
  You may label DNA, RNA, or oligonucleotides with digoxigenin (DIG).
- *Label the probe by a variety of familiar, efficient procedures.*
  The DIG labeling techniques are simple adaptations of the enzymatic labeling procedures you've used many times.
- *Use the probe to detect even rare DNAs or RNAs.*
  With DIG-labeled probes, you can easily detect single-copy genes on Southern blots, unique mRNAs on Northern blots, rare recombinants in bacterial colonies or viral plaques, or important nucleic acids *in situ*.

How can the DIG System be such a powerful tool, yet so simple to use? By combining familiar, proven techniques with innovative DIG technology.

DIG Labeling

The protocols for labeling DNA, RNA, and oligonucleotides with digoxigenin (DIG) (Figure 1) are based on well-established, widely used methods.

The methods (usually enzymatic) used to prepare radioactive probes may also be used to prepare DIG-labeled probes (Figure 2).

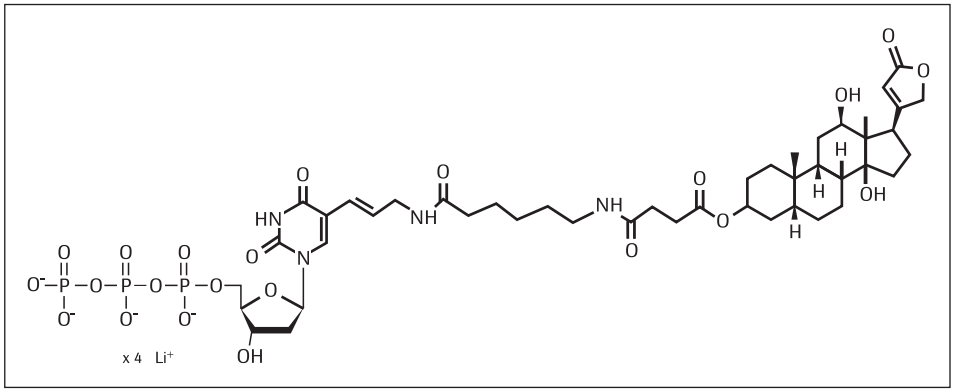

Figure 1. Structure of Alkali-labile Digoxigenin (DIG)-dUTP. This alkali-labile version of DIG-dUTP is used for most nonradioactive DNA labeling applications. It is included in many of our DIG DNA labeling kits. Other DIG-labeled nucleotides are available for nonradioactive labeling of RNA and oligonucleotides.

Roche has developed a complete line of kits that provide optimal incorporation and spacing of digoxigenin in nucleic acids. These include:

- **PCR DIG Probe Synthesis Kit,** for incorporation of DIG-11-dUTP into DNA by the polymerase chain reaction.
- **DIG-High Prime and DIG-High Prime DNA Labeling and Detection Starter Kits I and II,** for incorporation of DIG-11-dUTP into DNA by the random-primed labeling method.
- **DIG RNA Labeling Kit (SP6/T7) and DIG Northern Starter Kit,** for the incorporation of DIG-11-UTP into strand-specific RNA probes by RNA polymerase-mediated transcription.
- **DIG Oligonucleotide 3'-End Labeling Kit, 2nd generation** for addition of a single DIG-11-ddUTP to the 3'-end of an oligonucleotide (by terminal transferase).
- **DIG Oligonucleotide Tailing Kit, 2nd generation** for addition of a series of alternating DIG-11-dUTP and dATP to the 3'-end of an oligonucleotide (by terminal transferase).

In addition, we have developed optimized protocols for addition of DIG by nick translation or cDNA synthesis.

See Chapter 2, page 44, for a complete listing of DIG System kits and reagents.

Hybridization and Detection

Standard hybridization protocols (Figure 2) have been adapted to allow hybridization of digoxigenin-labeled probes to target nucleic acids (*e.g.* DNA on a Southern blot or RNA on a Northern blot). The only major change involves pretreatment of the membrane with a special blocking reagent to eliminate nonspecific background.

For detection of the hybridization signal on the nucleic acid blot (Figure 2), the DIG System has adapted immunochemical techniques that were developed for Western blots. First, an alkaline phosphatase conjugate of an anti-digoxigenin antibody binds to the hybridized probe. Then, the antibody-probe hybrids are visualized with chromogenic or chemiluminescent alkaline phosphatase substrates. Chromogenic substrates produce a colored signal directly on the membrane. More sensitive chemiluminescent substrates produce light that can be conveniently recorded with X-ray film (as with 32P or 35S-labeled probes).

We offer both individual reagents and reagent sets for the detection of DIG-labeled probes, including:

- **Anti-Digoxigenin-Alkaline Phosphatase,** for detection of DIG probe-target hybrids on membranes
- Other **Anti-Digoxigenin conjugates** (including Anti-Digoxigenin-Peroxidase, Anti-Digoxigenin-Fluorescein, and Anti-Digoxigenin-Rhodamine) suitable for visualization of DIG probe-target hybrids in *in situ* applications

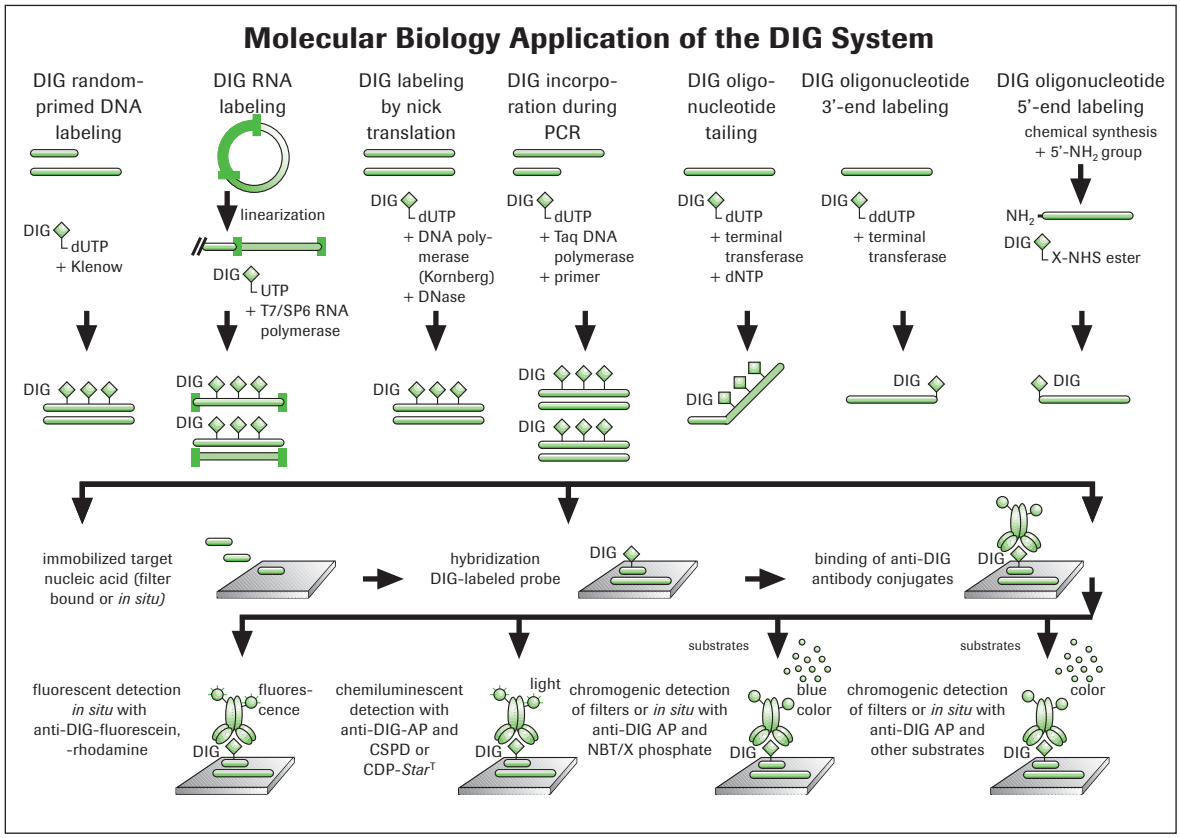

Figure 2. Labeling and Detection Alternatives Offered by the DIG System.

- **CDP-Star alkaline phosphatase substrate, CSPD alkaline phosphatase substrate, and DIG Luminescent Detection Kit for Nucleic Acids,** for sensitive chemiluminescent visualization of DIG-labeled probes.
- A variety of chromogenic alkaline phosphatase substrates for generation of colored precipitates directly on the membrane (at the site of the probe-target hybrids).

These substrates include:

- **DIG Nucleic Acid Detection Kit,** a convenient kit that uses NBT and BCIP
- **HNPP alkaline phosphatase substrate**

See Chapter 2, page 49, for a complete listing of DIG System substrates and antibodies.

1

An Overview of the DIG Application Manual

This DIG Application Manual can serve as a convenient reference for designing and performing nonradioactive labeling and membrane hybridization experiments.

The manual gives step-by-step instructions for the most commonly used digoxigenin labeling methods and alkaline phosphatase-based assays for detecting DIG-labeled probes on a membrane.

It also contains detailed procedures for:

- Single-copy gene detection on human genomic Southern blots
- Detection of unique mRNA species on Northern blots
- Colony and plaque screening
- Differential and array screening of cDNAs

In addition, this manual contains many tips for getting the best membrane hybridization results with the DIG System. For instance, it describes "estimation of probe yield" (page 85 in Chapter 3) for determining the correct amount of probe be used in the subsequent hybridizations.

The manual also contains a brief overview of other nonradioactive applications, including Western blot analysis, telomere assays, RNase protection assays, gel mobility shift assays, and direct detection of DIG-labeled DNA on a gel. See Chapter 4 in this guide for a description of these techniques.

The use of the DIG System for in situ hybridization is not discussed in this guide. For a comprehensive treatment of nonradioactive in situ hybridization applications, ask your Roche representative for a free copy of our "Nonradioactive In Situ Hybridization Application Manual".

How to Use the Manual

To get the most value from this manual, start at the beginning – read Chapter 2. In "DIG Basics," you will find an overview of all the steps in a typical DIG System procedure, from labeling the probe through stripping the probe off the membrane. For each step, the chapter provides:

- Information you need to choose the best DIG System techniques for your application
- Guidelines for experimental parameters (template concentration, target concentration, substrate, etc.) that will ensure the most sensitive results with the lowest background

When you finish reading Chapter 2, you should be able to design the correct labeling and detection experiment to get the results you need. You can then use the DIG Product Selection Guide (Chapter 2, page 44) to choose the kits and reagents you need.

After you have designed your system, you can follow the detailed DIG labeling and detection procedures in Chapter 3 to perform your experiment (Figure 3).

Tip: To quickly locate all the procedures you need, use the table, "Location of Procedures in this Guide" at the end of this introduction.

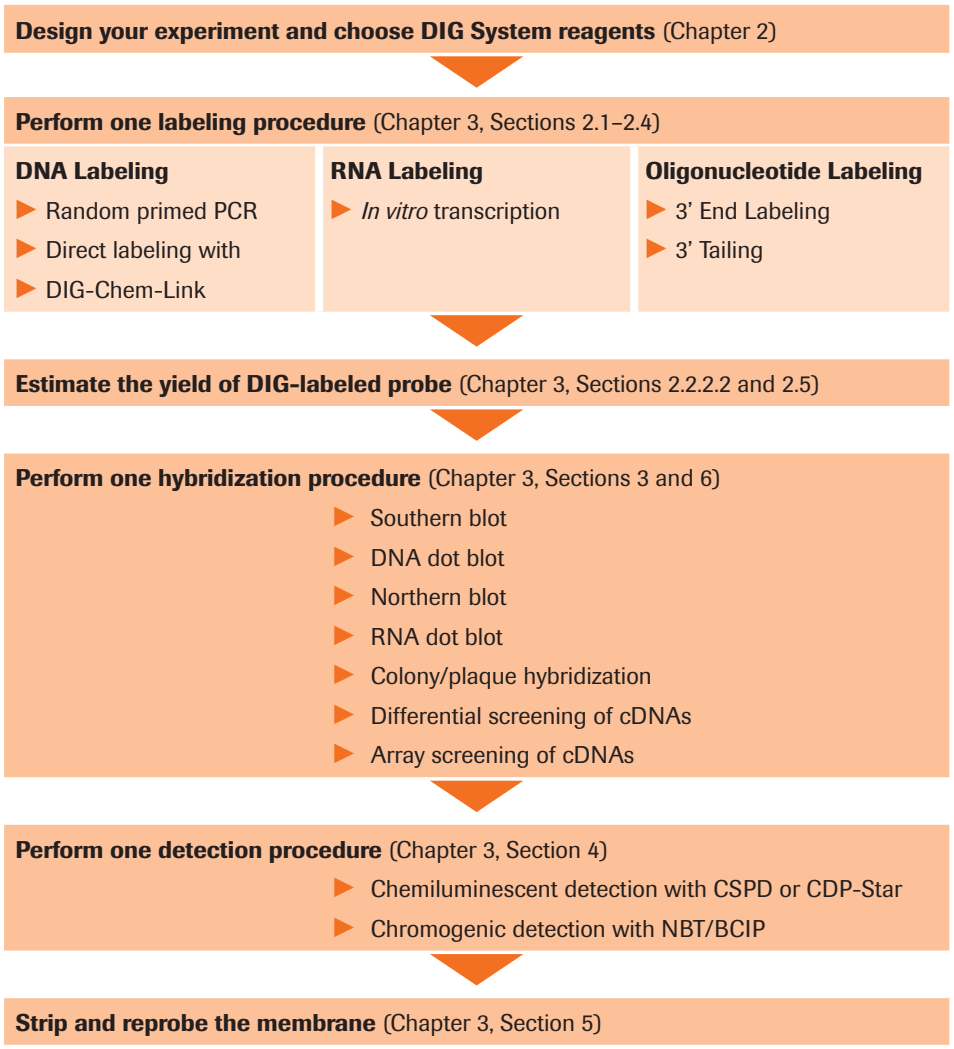

Figure 3. Flow Chart for Performing a DIG Labeling and Detection Experiment.

Location of Procedures in this Manual

Technique	Location of Procedure		
	Chapter	Section	Page
Labeling DNA			
Random primed labeling	3	2.1.2	60
PCR labeling	3	2.2.2	66
Nick translation	–	See Note 1	–
Labeling RNA			
In vitro transcription	3	2.3.2	78
Labeling oligonucleotides			
3' End labeling	3	2.4	84
3' Tailing	3	2.4	84
Estimating yield of labeled probe			
Direct detection	3	2.5	85
Gel electrophoresis	3	2.2.2.2	69
Hybridization of probe to target			
Southern blot	3	3.1.2	97
DNA dot blot	3	3.1.2	97
Northern blot	3	3.2.2	107
RNA dot blot	3	3.2.2	107
Colony/plaque hybridization	3	6.1.2	137
Differential screening of cDNAs	3	6.2.2	144
Array screening of cDNAs	3	6.2.2	145
In situ hybridization	–	See Note 1	–
Detection/visualization of probe-target hybrids			
Chemiluminescent assay	3	4.1.2	117
Chromogenic assay with NBT/BCIP	3	4.2.2.2	123
Stripping and reprobing membrane			
DNA blot, after chemiluminescent assay	3	5.2.1	129
DNA blot, after chromogenic assay	3	5.2.2–5.2.3	129
RNA blot, after chemiluminescent assay	3	5.2.4	130
Other techniques			
"Mock hybridization" for minimizing background	5 B	2	195
Troubleshooting membrane background problems	5 B	1	165
Miscellaneous procedures for improving results	5 C	–	177

1 Detailed procedures are not given in this guide, but are available in our „Nonradioactive *In Situ* Hybridization Application Manual". Ask your local Roche representative for a copy of this *in situ* hybridization manual.

2

DIG Basics

2

1. Introduction

The DIG Nonradioactive System offers a very powerful and sensitive method for nucleic acid labeling and detection. This chapter gives a brief overview of the different steps involved in producing a DIG-labeled hybridization probe (DNA, RNA, or oligonucleotide) and using that probe to detect target nucleic acid (RNA or DNA) on a membrane (Southern, Northern, or dot blot).

The "DIG Basics" information in this chapter will help you design or improve your DIG labeling and detection system.

Once you have decided the best way to perform each step in **your** DIG system, you can turn to Chapter 3 of this manual, where you can get detailed procedures and tips for each step.

For your convenience, we have included a "quick reference" version of the "DIG Basics" information in Chapter 5 A, page 160 of this manual. Turn to that appendix for a quick overview of the information you need to design your DIG system.

Topics in this section include:

2. DIG Labeling

This section is designed to help you choose the labeling method that is best for your purposes. The section includes:

- A summary table showing the many labeling methods available, the amount of template they require, the sensitivity of the probes produced by the method, the applications for which the probes are suited, and the labeling kit or reagent that Roche offers for that method.

  Scientists in our laboratories and others have developed techniques for optimizing the various DIG labeling methods. For information on optimization as well as detailed procedures for the labeling methods, see the pages indicated in the last column of the table.

- A comparison of the three commonly used labeling methods: PCR labeling, random primed labeling, and RNA labeling. This table should help you choose between these three popular labeling methods.

2

2.1 Overview of Nonradioactive DIG Labeling Methods

Method (Roche Kit Available)	Advantages of Method	Amount of Starting Template Required	
PCR Labeling (PCR DIG Probe Synthesis Kit, Cat. No. 11 636 090 910)	▶ Requires only a small amount of template ▶ Even impure templates can be used ▶ Requires less optimization than other methods ▶ High yield of labeled probe ▶ Recommended for very short probes (<100 bp) ▶ Produces very sensitive probes	▶ Plasmid DNA, 10 – 100 pg (ideally, use 10 pg) ▶ Genomic DNA, 1 – 50 ng (ideally, use 10 ng)	
Random Primed Labeling, high efficiency [DIG-High Prime DNA Labeling and Detection Starter Kits I (Cat. No. 11 745 832 910) **and II** (Cat. No. 11 585 614 910)]	▶ Produces very sensitive probes ▶ Reaction can be scaled up tenfold	▶ 300 ng DNA (for probes that can detect single copy genes) ▶ Lower amounts of template may be used if labeling reaction is performed overnight	
Nick Translation	▶ Allows control of probe length (important in *in situ* hybridization applications)	1 µg DNA	
RNA Labeling (DIG Northern Starter Kit, Cat. No. 12 039 672 910**)**	▶ Generates large amounts of probe ▶ Labeled probe is completely free of vector sequences ▶ RNA probes are single-stranded ▶ RNA probes are more sensitive than DNA probes for analyzing northern blots ▶ DIG-labeled RNA probes can easily be fragmented for *in situ* hybridization	▶ Plasmid DNA, linearized: 1 µg ▶ PCR product with promoter: 100 – 200 ng	
3' End Labeling (DIG Oligonucleotide 3' End Labeling Kit, 2nd generation Cat. No. 03 353 575 910)	▶ Requires only a small amount of template ▶ Reaction can be scaled up indefinitely	100 pmol oligonucleotide	
3' Tailing (DIG Oligonucleotide Tailing Kit, 2nd generation Cat. No. 03 353 583 910)	▶ Requires only a small amount of template ▶ Produces more sensitive probes than end labeling ▶ Reaction can be scaled up indefinitely	100 pmol oligonucleotide	

2

	Labeled Probe Can Detect	Labeled Probe May Be Used For	Comments	For Detailed Procedure, see Page
	0.10 – 0.03 pg DNA	▶ Genomic Southern blots ▶ Northern blots (Also works in library screening, dot/slot blots, and *in situ* hybridizations)	▶ Optimize PCR in the absence of DIG-dUTP before attempting labeling reaction ▶ Template concentration is crucial to producing sensitive probes ▶ Use less DIG to label long (>1 kb) probes ▶ Do not substitute the PCR DIG Labeling Mix (DIG-dUTP: dTTP = 1:20) for the labeling mix (DIG-dUTP: dTTP = 1:3) provided in this kit.	64 (Chapter 3) *For a comparison of this and other labeling methods, see page 20 of this chapter.*
	0.10 – 0.03 pg DNA	▶ Genomic Southern blots ▶ Library screening (Also works in dot/slot blots and Northern blots)	▶ Requires highly purified template ▶ Very sensitive to template impurities ▶ Resuspend purified template in H_2O or Tris buffer. Do not use TE, because EDTA will inhibit labeling reaction.	57 (Chapter 3) *For a comparison of this and other labeling methods, see page 20 of this chapter.*
	Mainly used for *in situ* applications; sensitivity depends on target system	▶ *In situ* hybridization	▶ Requires highly purified template ▶ Template should not be denatured before it is labeled.	–
	0.10 – 0.03 pg DNA 0.10 – 0.03 pg RNA	▶ Northern blots ▶ *In situ* hybridization ▶ Library screening ▶ Dot/slot blots	▶ Requires highly purified template	74 (Chapter 3) *For a comparison of this and other labeling methods, see page 20 of this chapter.*
	10 pg DNA	▶ Library screening ▶ Dot/slot blots ▶ *In situ* hybridization	▶ Oligos should be purified by HPLC or Elutips (Schleicher and Schuell) ▶ Probes can be used without purification	84 (Chapter 3)
	1 pg DNA	▶ Library screening ▶ Dot/slot blots ▶ *In situ* hybridization	▶ Oligos should be purified by HPLC or Elutips (Schleicher and Schuell) ▶ Probes can be used without purification	84 (Chapter 3)

2

2.2 Comparison of PCR Labeling, Random Primed Labeling, and RNA Labeling

This section gives a brief overview of the similarities and differences between PCR labeling, random primed labeling, and RNA labeling methods.

The information in this section should help you choose between these three popular methods for adding a DIG label to a hybridization probe.

How the Three Methods Work

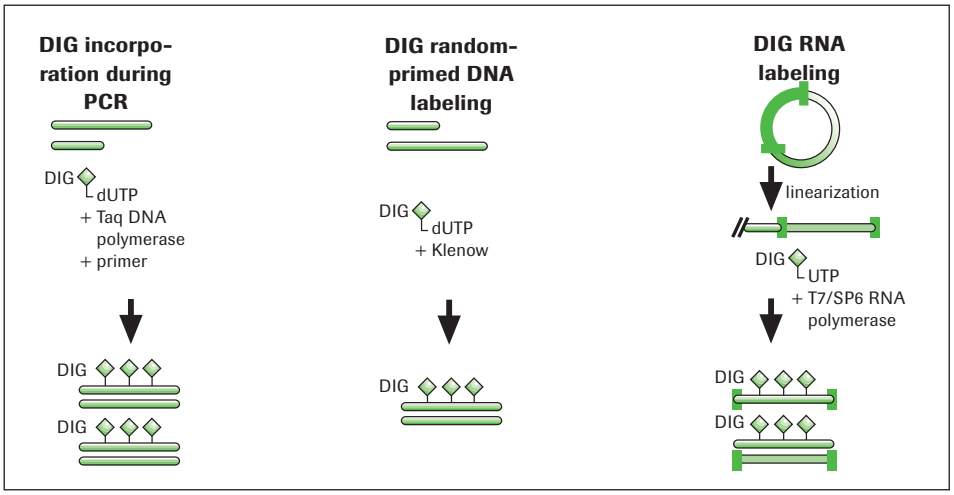

Figure 4: PCR Labeling, Random Primed Labeling, and RNA Labeling

2.2.1 A Closer Look at PCR Labeling

In PCR, a thermostable polymerase (*e.g.* Taq polymerase) can quickly amplify a specific DNA target over a millionfold. If the reaction mixture contains DIG-dUTP, the amplified DNA becomes a highly labeled, very specific, and very sensitive hybridization probe. The specificity of the PCR labeling reaction makes this technique especially suitable for labeling very short target sequences.

PCR-labeled probes are especially suitable for single copy sequence detection on genomic Southern blots and rare mRNAs on Northern blots. Of course, they also work for library screening, dot/slot blots, and *in situ* hybridizations.

PCR labeling produces a very high yield of labeled probe from very little template. The minimum and maximum amount of template for PCR labeling ranges from 10 to 100 pg plasmid and from 1 to 50 ng genomic DNA. However, our experience indicates that using only 10 pg plasmid or 10 ng genomic DNA leads to optimal results. In general, cloned plasmid inserts give better results than genomic DNA.

The sequence of the PCR primers determines what region will be amplified and labeled. That means the quality of the template preparation does not normally influence the PCR labeling reaction. Even very crudely purified plasmid preparations (*e.g.* prepared by boiling) may be used. It also means that PCR labeling requires less optimization than other labeling methods.

2.2.2 A Closer Look at Random Primed Labeling

Random primed labeling can label templates of almost any length.

For very short sequences, use the PCR labeling method (Section 2.2.1 above) for best results.

In random primed labeling, Klenow enzyme copies DNA template in the presence of hexameric primers and alkali-labile DIG-dUTP. On average, the enzyme inserts one DIG moiety in every stretch of 20 – 25 nucleotides. The resulting labeled product is a homogeneously labeled, very sensitive hybridization probe (able to detect as little as 0.10 – 0.03 pg target DNA).

The spacing of the DIG molecules is very important. If DIG molecules were closer to each other, steric hindrance would prevent the large anti-DIG antibody from recognizing and binding the labeled probe.

These labeled probes are especially suitable for single copy gene detection on genomic Southern blots and in screens of recombinant libraries. Of course, they also work for dot/slot blots, and Northern blots.

Since each primer has a different six-base sequence, the labeled probe product will actually be a collection of fragments of variable length. Thus, the labeled probe will appear as a smear, rather than a unique band on a gel. The size distribution of the labeled probe depends on the length of the original template.

Example: *Labeled fragments generated from a 2 kb template will range from approximately 300 – 1500 bp with most being approximately 800 bp long.*

Unlike PCR labeling, random primed labeling requires highly purified templates [preferably prepared by the High Pure Plasmid Isolation Kit (Cat. No. 11 754 777 001 or 11 754 785 001) or density gradient (CsCl) centrifugation].

2.2.3 A Closer Look at RNA Labeling

Labeled RNA probes are generated by *in vitro* transcription from a linearized DNA template. During the transcription, many RNA copies of the DNA product are made and each is labeled with DIG-UTP. The yield of the probe from the RNA labeling reaction is very high. The labeled sequence is unique and highly labeled.

The unique template sequence must be downstream from a viral promoter (*e.g.* for T7, SP6, or T3 RNA polymerase).

The template may be either highly purified, linearized plasmid DNA or a PCR product which has had a suitable promoter added during amplification.

Labeled RNA probes are very sensitive. In fact, DIG-labeled RNA probes offer much better sensitivity than DIG-labeled DNA probes for detecting RNA targets. Thus, RNA probes are especially suitable for detection of rare mRNAs on Northern blots. Of course, they also work for Southern blots, library screening, dot/slot blots, and *in situ* hybridizations.

2

2.2.4 Summary Table: Comparison of Important Parameters for the Three Labeling Methods

Parameter	PCR Labeling	Random Primed Labeling	RNA Labeling
Amount of template required to produce probes that can detect single-copy genes	▶ 10 pg plasmid, or ▶ 10 ng genomic DNA	▶ 300 ng – 1 µg genomic DNA or plasmid	▶ 1 µg linearized DNA, or ▶ 100 – 200 ng PCR product with promoter
Preferred template for best results	▶ Cloned plasmid insert, unpurified [Even plasmids prepared by "quick preparation" methods (mini-preps, maxi-preps) or by simple boiling of cells may be used.]	▶ Cloned plasmid insert, highly purified (Preferably, prepare plasmid with the High Pure Plasmid Isolation Kit, Cat. No. 11 754 777 001, or use density gradient centrifugation in the presence of cesium chloride.)	▶ Specially prepared PCR product (must contain SP6, T3, or T7 RNA promoter), purified ▶ Preparation must be free of EDTA and RNases ▶ Optimal template length: 0.3 – 1.0 kb ▶ Template must be linearized with a restriction enzyme that leaves a 5' overhang ▶ Template should be extracted with phenol/chloroform after restriction digest to ensure elimination of RNases
Purity of template required	▶ Low	▶ High	▶ High
Optimal ratio, DIG-dUTP:dTTP	▶ 1:3 (for probes <1kb long), or ▶ 1:6 (for probes 1 – 3 kb long), or ▶ From 1:6 to 1:10 (for probes >3 kb long)	▶ 1:3	▶ 1:3
Length of labeled product	▶ Unique (dependent on primers)	▶ Variable (dependent on original length of template)	▶ Unique (dependent on promoter and termination sites)
Nature of labeled product	▶ Many copies of a unique labeled DNA	▶ Multiple labeled DNAs, transcribed from fragments of original template	▶ Many copies of a unique labeled RNA
Sensitivity of labeled probe	▶ 0.10 – 0.03 pg DNA	▶ 0.10 – 0.03 pg DNA	▶ 0.1 – 0.03 pg RNA or DNA

Conclusions: The most flexible and powerful way to add DIG label to DNA is by PCR, particularly if highly purified template is not available. Random primed labeling will produce probes of equal sensitivity if sufficient highly purified template is available. DIG-labeled RNA gives the highest sensitivity for detection of target RNA.

3. Evaluation of Probe Labeling Efficiency

It is important to check the efficiency of each labeling reaction by determining the amount of DIG-labeled product. This will enable you to add the correct amount of probe to the hybridization solution.

The consequences of using the wrong amount of probe in a hybridization are severe.

Too much probe will lead to serious background problems. Too little probe will lead to little or no hybridization signal. (For details, see Chapter 5 B, page 168)

The table below shows the preferred technique for estimating the amount of labeled probes prepared by different methods:

Preferred Method for Estimation of Labeled Product

Labeling Method	Preferred Estimation Technique
Random Primed labeling	Direct detection
PCR	Gel electrophoresis
Nick Translation	Direct detection
RNA probes	Direct detection[1]
3' End-labeling (oligonucleotides)	Direct detection
3' Tailing (oligonucleotides)	Direct detection

[1] RNA probes may be examined for integrity, but not quantified, by gel electrophoresis.

3.1 Overview of Direct Detection Procedure

The preferred way to roughly quantify almost all labeled nucleic acid probes (except PCR-labeled probes) is the direct detection method. In this method, a series of dilutions prepared from the DIG-labeled probe is spotted directly on a membrane and visualized with standard DIG detection procedures (Figure 5).

For comparison, known concentrations of a DIG-labeled control nucleic acid are spotted on the same membrane.

For a list of available DIG-labeled controls, see Chapter 3, page 85.

2

Step[1]		Performed with this Solution[1]	Time Required
1	Spot probe and control on nylon membrane.	Serial dilutions of labeled experimental probe and known DIG-labeled control nucleic acid	15 min
2	Wash membrane briefly.	Washing Buffer	2 min
3	Block the membrane.	1× Blocking Solution	30 min
4	Let the antibody bind DIG label on the membrane.	1:10 000 dilution of Anti-DIG-alkaline phosphatase in 1× Blocking Solution	30 min
5	Wash the membrane twice to remove unbound antibody.	Washing Buffer	2× 15 min
6	Equilibrate the membrane.	Detection Buffer	2 min
7	Place membrane in bag, add chemiluminescent reagent, and seal bag.	Ready-to-use CSPD or CDP-Star[2]	5 min
8	(Optional) Preincubation/ activation of CSPD.	Ready-to-use CSPD (not necessary with CDP-Star)	(10 min)
9	Expose the sealed bag (containing the membrane) to X-ray film.	-	5 – 25 min
			Total time required: 2.0 h (CDP-*Star*)
			2.5 h (CSPD)

Figure 5. Flow Diagram of Direct Detection Procedure

[1] For details of these steps and solutions, see Section 2.5, page 85 of Chapter 3.
[2] Alternatively this procedure can be performed with NBT/BCIP, see DIG Starter Kit I.

If you see a signal from the spot that contains 0.1 pg of your probe (Figure 6), your probe has reached full sensitivity.

The labeling reaction produced an optimal yield of the labeled probe.

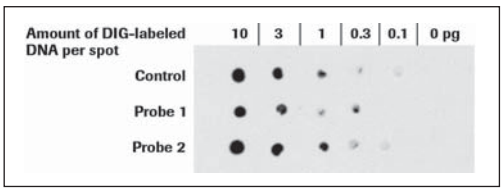

Figure 6. Results of Direct Detection Procedure. Two different DIG-labeled probes were prepared (each from 1 µg template) by random primed labeling. Dilutions of each were spotted on a nylon membrane and analyzed by the direct detection procedure.
For reference, dilutions of a DIG-labeled control DNA were included on the same membrane. Chemiluminescent alkaline phosphatase substrate (CDP-*Star*) was used to visualize the DIG signal in the spots. Exposure time was 10 min. The amount of DIG-label DNA in each spot (estimated from the amount of starting template) is shown at the top of the figure.

Result and conclusion: The 0.3 pg spot of each DNA is visible. However, only probe 2 and the control DNA contain sufficient label to give a signal in the 0.1 pg spot. Therefore, probe 2 has reached full sensitivity. That probe can be used (at the concentration recommended in the procedure) to detect, for instance, single-copy genes on a genomic Southern blot. Note that probe 1 is also sensitive enough for detection of single copy genes on a genomic Southern blot, but you may have to use a greater portion of the labeled product in each hybridization than you will with probe 2.

2

3.2 Analysis of PCR-labeled Probes by Gel Electrophoresis

The labeling efficiency for PCR-labeled probes can be determined without a direct detection procedure. Instead, a quick estimate of labeling efficiency can be performed by gel electrophoresis, as described below. Evaluation of PCR-labeled probes on a gel requires significantly less time than evaluation of probes by the direct detection method.

For a detailed description of the gel electrophoresis assay, see Chapter 3, page 95.

To estimate the labeling efficiency of your PCR-generated probe, you must have included a reaction in your PCR that produces an unlabeled version of your experimental probe (*i.e.*, amplified in the absence of DIG-dUTP).

Run a portion (5 µl) of each PCR product (both unlabeled and DIG-labeled versions of your experimental probe) on a mini-gel. When you stain the gel with ethidium bromide (Figure 7), you should note the following:

1. DIG-labeled probe should apparently be significantly larger than unlabeled probe.

   The presence of DIG in DNA makes it migrate in a gel more slowly than a corresponding unlabeled piece of DNA of the same size.

2. Unlabeled probe DNA should run at its predicted molecular weight.
3. Intensity of the stained DIG-labeled probe should be equal to (or slightly less than) the intensity of the unlabeled probe DNA.
4. The presence of DIG in the reaction mixture has slowed the polymerase and reduced the labeling efficiency of the reaction.

If all these conditions are met, the probe was labeled efficiently. Consequently, you can use the standard amount of labeled probe (2 µl of PCR product per ml hybridization buffer) in hybridization reactions.

If you also run a known amount of a DIG-labeled molecular weight marker (available from Roche) on the same gel, you can get a rough estimate of the amount of DIG-labeled experimental probe from:

- The relative intensity of your labeled experimental probe and a band in the molecular weight marker that is similar in size.
- The (calculated) amount of that marker band on the gel.

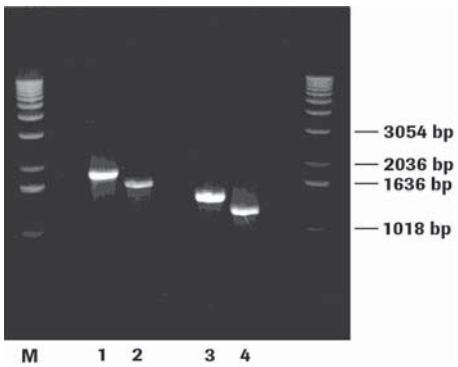

Figure 7. Evaluation of PCR-labeled Probes by Agarose Gel Electrophoresis. On this agarose gel, the labeled PCR products have a significantly greater molecular weight than the unlabeled products. (This is due to high density labeling with DIG.)

Lanes 2 and 4: unlabeled PCR product.

Lanes 1 and 3: corresponding DIG-labeled PCR products.

M: molecular weight marker.

Result and conclusion: The labeled and unlabeled products migrate as separate bands on an electrophoretic gel. If you see this shift in molecular weight and the intensity of the labeled band is equal to (or slightly less than) the intensity of the unlabeled product, your labeling reaction was successful. You can use the standard amount of labeled probe (2 µl of PCR product per ml of hybridization buffer) in your hybridization experiments.

2

4. Electrophoresis of Target Nucleic Acids on Agarose Gels

The most common use for a DIG-labeled probe is the detection of specific nucleic acid sequences on a blot (*e.g.*, Southern, Northern, dot). For best results, the target nucleic acids should be electrophoretically separated on agarose and blotted under optimal conditions.

This section summarizes guidelines for optimizing the electrophoretic separation of nucleic acids. The next section will discuss ways to optimize the blotting process.

4.1 Amount of Target Nucleic Acid To Load

For typical experiments, use the guidelines in this table to determine how much target nucleic acid to load on the agarose gel:

Amount of Target Nucleic Acid To Load on an Electrophoretic Gel

To Analyze This Target Load	This Amount of Target (per lane, each sample)	When You Are Using This Probe
▶ Genomic DNA	1.0, 2.5, 5.0 µg (3 separate samples)	DIG-labeled DNA or RNA
▶ Plasmid DNA	<1 ng	
▶ Total RNA	≤1 µg	DIG-labeled RNA
▶ mRNA	≤100 ng	
▶ Total RNA mRNA	≤5 µg	DIG-labeled DNA
	≤500 ng	

4.2 Pouring and Running the Agarose Gel

Follow these guidelines for preparing and running all gels:

- All gels should be prepared fresh just before use.
- If using thin gels, use the gel within 1 h after pouring.

  ⚠ *Thin gels will suffer drying effects if left for longer than 1 h.*

- Do not include ethidium bromide in the gel, because ethidium can cause uneven background if the gel is not run long enough.

  ❗ *After electrophoresis, stain the gel with 1 µg/ml ethidium bromide and destain with water (RNase-free if the gel contains RNA) to ensure that the target nucleic acid is intact.*

For more information on gels, see Chapter 5, page 181.

4.2.1 Separating RNA on Agarose Gels

RNA gels require extra care. Follow these guidelines:

- Prevent RNase contamination of the work area by doing the following:
  - Always wear gloves during the procedure,
  - Bake all glassware before use, and
  - Wipe all gel preparation areas of the lab bench with a decontaminating solution.
- All gel reagents must be free of RNase. Either purchase RNase-free reagents or treat the reagents with DMDC (dimethyl-dicarbonate) or DEPC (diethyl pyrocarbonate) to eliminate RNase contamination.

  Unless you can purchase RNase-free glycerol, omit glycerol from the sample loading buffer, since glycerol cannot be successfully treated to remove RNases.

  Do not assume that commercially available sample loading buffers which contain glycerol are RNase-free.
- For best results when separating RNA, omit glycerol from the sample loading buffer and use a dry load procedure to start the gel, as follows:
  - Prepare a glycerol-free sample buffer that contains denaturants, buffer, and marker dyes (*e.g.*, formamide, formaldehyde, MOPS buffer, xylene cyanol, and bromophenol blue).
  - Incubate the samples at 65°C for 10 min to denature the RNA, then chill on ice.
  - Pour RNase-free running buffer into the electrophoresis chamber only to the rim of the gel, but not over the sample wells.
  - Load the glycerol-free samples onto the dry gel.
  - Carefully fill the sample wells to the top with running buffer.
  - Start the electrophoresis (100 V).
  - Once the samples have run into the gel (about 10 min), stop the electrophoresis and add more running buffer to the electrophoresis chamber until the gel is completely covered.
  - Reduce the voltage and allow the gel to run as usual.
  - Reduce the formaldehyde concentration to 2% in the RNA gels. These low formaldehyde gels produce higher resolution and sharper bands.

4.2.2 Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) is much more complicated than standard agarose gel electrophoresis. The separation conditions are tricky and blotting extremely large DNA fragments is not trivial. Yet, if the PFGE is performed with care and the blot transfer of nucleic acid is flawless, the DIG detection system has the sensitivity to detect unique sequences on the blot.

For more information of the PFGE technique, see a standard laboratory manual such as Molecular Cloning, A Laboratory Manual (Sambrook, Fritsch, and Maniatis).

2

5. Blot Transfer of Target Nucleic Acids to a Membrane

The table below should help you choose the best blot transfer method for your appliction. For the DIG system, we prefer the capillary transfer method for standard Southern and Northern blots.

For more tips on treatment of gels before blot transfer, see Chapter 5 C, page 181.

Characteristics of Different Blot Transfer Methods

Transfer Method	Pretreatment of Gel Before Blotting	Amount of Salt
Capillary Transfer	**Southerns**: Depurination (if target ≥5 kb), denaturation, neutralization **Northerns**: Soak 10 min in 20× SSC to remove formaldehyde	20× SSC transfer buffer (RNase-free for northerns)
Dry Blot	Soak in 20× SSC, 20 – 30 min	No transfer buffer required
Contact Blot	None required *This procedure is used only when the assay allows direct detection (e.g. as in the RNase protection assay). It should **not** be used for regular Southern or Northern blots*	No transfer buffer required
Electroblotting	Equilibrate in transfer buffer	According to recommendations of manufacturer
Vacublotting	Same as capillary transfer method.	20× SSC for DNA or RNA

Technology	Reasons for Choosing Method
▶ One-way, passive transfer of target in buffer *For details of capillary transfer method, see Section 3.1.2.2, page 97 of Chapter 3 (for DNA) or Section 3.2.2.2, page 107 of Chapter 2 (for RNA).*	▶ Provides highest sensitivity ▶ Very reliable and reproducible ▶ Incomplete transfer will be evident before hybridization
▶ One- or two-way capillary transfer performed without using transfer buffer – uses buffer present in the gel ▶ Can be done in both directions (sandwich blot)	▶ Good for high agarose concentrations (*e.g.* gel shift assays with 4 – 6% gels or separation of oligonucleotides on up to 10% gels) ▶ Allows preparation of two blots from one gel
▶ Form of dry blot without the presoaking step ▶ Performed without transfer buffer – uses buffer present in the gel *Transfer must start immediately after electrophoresis or DNA will dry in the gel and transfer will not occur.*	▶ Good for sequencing gels ▶ Ideal for 0.2 mm thick gels *Thickness of gel should not exceed 0.2 mm.* ▶ Short transfer time (approx. 20 min) ▶ Can be used for all experiments that use a direct detection assay (*e.g.* RNase protection assay)
▶ Uses electrical current to speed transfer *Only recommended for polyacrylamide gels.*	▶ Rapid transfer
▶ Uses vacuum to speed transfer *Blot will be destroyed if vacuum breaks down. Transfer will not be complete.* ▶ Often used with alkaline transfer (0.4 M NaOH as transfer buffer) (for DNA only) *Alkaline method has been reported to lead to reduced sensitivity with the DIG system. No crosslinking is possible after alkaline transfer.*	▶ Rapid transfer (approx. 1 hr) *Not as sensitive as capillary blotting.*

2

5.1 Comments on Blot Membranes

The type of membrane used for the blot influences both the sensitivity and the background of the DIG assay. Available choices and their characteristics are given in this table:

Characteristics of Different Types of Blot Membranes

Membrane Type	Application	Comments
Positively Charged Nylon, from Roche	Suitable for either colorimetric or chemiluminescent detection	▶ More durable than nitrocellulose ▶ Higher sensitivity than nitrocellulose ▶ Higher target binding capacity than uncharged nylon membranes ▶ Positive charge is controlled to ensure low background, maximum signal-to-noise ratio ▶ Tested with the DIG system
Positively Charged Nylon, from other suppliers	Suitable for either colorimetric or chemiluminescent detection	▶ Great variability in background, lot-to-lot and brand-to-brand, due to variability in positive charge on membrane *Most suppliers assay charge density only to establish a minimum charge. They do not test for an upper limit. Thus, one lot from a supplier might be just above the minimum charge density and give good results, while the next lot has a hundredfold greater charge density and gives a much higher background.*[1]
Uncharged Nylon	Suitable for either colorimetric or chemiluminescent detection	▶ Lower target binding capacity than positively charged nylon membranes
Nitrocellulose	Suitable for colorimetric detection assays *Gives lower sensitivity than nylon membranes.*	▶ Regular nitrocellulose cannot be used for chemiluminescent detection assays, since a hydrophobic surface is required for chemiluminescence assay *A special hydrophobic nitrocellulose (Nitro-Block) is available from Tropix and can be used in a chemiluminescent assay. However, the sensitivity will be ten- to a hundredfold less than possible with nylon membranes.* ▶ DNA cannot be UV crosslinked to membrane

[1] To compensate for lot-to-lot variations of charge in nylon membranes from other suppliers, always use our DIG Easy Hyb hybridization buffer. DIG Easy Hyb can minimize the background problems caused by the variability in charge density, resulting in lower background.

6. Hybridization of Probe to Target

The guidelines in this section will help you choose the best conditions for hybridizing DIG-labeled probe to target nucleic acid on a blot.

Comment: *DIG-labeled probes and ^{32}P-labeled probes behave with similar kinetics. They may be used under similar hybridization conditions.*

6.1 Concentration of DIG-labeled Probe During Hybridization

Recommended concentrations of various types of DIG-labeled probes

Probe Type	Probe Concentration During Hybridization
Random primed labeled DNA	25 ng/ml
PCR-labeled DNA	2 µl of a standard PCR labeling reaction/ml
DNA labeled with DIG-Chem-Link	20 – 50 ng/ml
Transcriptionally labeled RNA	20 – 100 ng/ml
RNA labeled with DIG-Chem-Link	50 – 100 ng/ml
End labeled oligonucleotide	1 – 10 pmol/ml
Tailed oligonucleotide[1]	0.1 – 10 pmol/ml

[1] For tailed oligonucleotide, add 0.1 mg/ml poly(A) and 5 µg/ml poly(dA) to the prehybridization buffer and the hybridization buffer. Poly(A) and poly(dA) prevent nonspecific signals that can be generated by the tails.

6.2 Determining an Optimal Hybridization Temperature

Always consider environmental factors, sequence-related factors, and type of hybrid when determining the optimal hybridization temperature. The effect of these factors is summarized below.

2

6.2.1 Three Environmental Factors That Influence Stringency

For any hybridization, stringency can be varied by manipulation of three factors (temperature, salt concentration, and formamide concentration):

Factor	Influence
Temperature	▶ High temperature increases stringency ▶ Low temperature decreases stringency
Salt concentration	▶ High salt decreases stringency ▶ Low salt increases stringency
Formamide	▶ Decreases melting point of DNA, thus lowering the temperature at which a probe-target hybrid forms *Adding 1% formamide lowers the melting temperature by 0.72°C.*

Example: *A combination of* ***high*** *temperature and* ***low*** *salt increases the hybridization stringency.*

6.2.2 Influence of Different Nucleic Acid Hybrids on Hybridization Temperature

The relative strength of different hybrids is:

RNA:RNA hybrids > RNA:DNA hybrids > DNA:DNA hybrids

Conclusion: *Hybrid types will influence the hybridization temperature used. RNA:RNA and RNA:DNA hybrids will require higher hybridization temperatures than DNA:DNA hybrids.*

Example: *For mammalian targets which contains 40% GC sequences, the optimal hybridization temperatures for each type hybrid in the presence of DIG Easy Hyb (or 50% formamide) are:*

For This Type Hybrid	T_{opt}* Is
DNA:DNA	37–42°C
DNA:RNA	50°C
RNA:RNA	68°C

**For all these hybrids, T_{opt} will vary according to the GC content of the probe and homology to the target. For example, T_{opt} for DNA:DNA varies between 37° and 42° for hybrids of 40 – 50% GC content and 80 – 100% homology to target.*

6.2.3 Influence of Sequence-related Factors on Hybridization Temperature

In addition to the environmental factors listed above, the homology of the probe to the target and the GC content of the probe influence the optimal hybridization temperature.

To determine the optimal hybridization temperature (T_{opt}), first calculate the melting temperature (T_m) of the probe-target hybrid, then set T_{opt} to a value that is 20° – 25°C below the calculated T_m.

For examples of T_{opt} calculations, see "Hybridization of Probe to Target," Chapter 5 A, page 162.

6.3 Components of the Hybridization Buffer

Many hybridization solutions are compatible with the DIG system. For instance, you could use any of the buffers listed in this table:

Suitable Hybridization Buffers for Use with the DIG System[1]

DIG Easy Hyb	High SDS Buffer	Standard Buffer + Formamide	Standard Buffer
Cat. No. 11 603 558 910 (see below)	7% SDS 50% Formamide, deionized 5× SSC 2% Blocking Reagent[2] 0.1% (w/v) N-lauroylsarcosine 50 mM Sodium phosphate, pH 7.0	50% Formamide, deionized 5× SSC 0.1% (w/v) N-lauroylsarcosine 0.02% (w/v) SDS 2% Blocking Reagent	5× SSC 0.1% (w/v) N-lauroylsarcosine 0.02% (w/v) SDS 1% Blocking Reagent

[1] Listed left to right, in order of preference.

[2] Blocking reagent is a purified fraction of milk powder, reconstituted and diluted in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, adjust with NaOH (solid) to pH 7.5 (20°C).

Our experience indicates the best choice for hybridization is our DIG Easy Hyb (Cat. No. 11 603 558 910). We recommend DIG Easy Hyb because it:

- Lowers the melting point by the same amount (36°C) as 50% formamide (*i.e.* by 0.72°C per 1% formamide) (Lower hybridization temperatures mean more reproducible results.)
- Is non-toxic and safe to use, since it does not contain formamide

  In place of formamide, DIG Easy Hyb contains enough urea to lower the hybridization temperature as though it contained 50% formamide.
- Is guaranteed to be sterile, as well as DNase- and RNase-free, so it will not degrade your template or probe
- Has been tested with nylon membranes for all types of blot applications, to ensure optimal results with the DIG System
- Is a ready-to-use hybridization buffer; it requires no additions
- Can conveniently be stored at room temperature
- Can help compensate for lot-to-lot variations in membranes from other manufacturers (See Section 5.1 on page 30 of this chapter for details.)

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6.3.1 Effect of Additives

Additives to the hybridization buffer have been reported to increase hybridization sensitivity of ^{32}P-labeled probes. For example, adding 10% dextran sulfate or 6 – 10% PEG 8000 can increase the hybridization sensitivity two- to threefold.

However, according to our observations, adding dextran sulfate or PEG to DIG Easy Hyb buffer does not increase the sensitivity of DIG-labeled probes. In addition, we do not recommend use of dextran sulfate and PEG 8000 with the DIG System, because there is considerable lot-to-lot variation in both products. Bad lots will lead to high background.

As a general rule, do not add any chemical to DIG Easy Hyb; use it as supplied for best results in nonradioactive hybridization experiments.

6.4 Hybridization Time Required

The time required for adequate probe-target hybridization depends upon the type of probe and the sensitivity required. The following table gives some general guidelines for hybridization times when DIG Easy Hyb is used as prehybridization and hybridization buffer:

Typical Hybridization Times[1]

Application	Recommended Hybridization Time[2]	For Example, see Page
DNA:DNA hybridizations		
Single copy gene detection	ON (12 – 16 h)	176
DNA fingerprinting (multiple locus probes)	2 – 4 h	––
DNA fingerprinting (single locus probes)	ON (12 – 16 h)[3]	140
Colony/Plaque hybridization	At least 4 – 6 h (up to ON)	
RNA:RNA hybridization	At least 4 – 6 h (up to ON)	121
Oligo:DNA hybridization	At least 1 – 6 h (up to ON)	–

[1] Prehybridization time for all applications (without probe) = 30 min.

[2] ON = overnight.

[3] Complete in 4 – 6 h with DIG Easy Hyb.

7. Stringent Washes of Blot

Two-stage stringent washes are performed after hybridization to disrupt undesired hybrids:

- **First Washes:** Low stringency (high salt concentrations and low temperatures), to remove nonspecifically bound probe.

The recommended low stringency wash for the DIG System is:

Type of Hybrid	Low Stringency Washes
All	▶ 2× 5 min in 2× SSC containing 0.1% SDS at room temperature

- **Second Washes:** High stringency (low salt concentrations and high temperatures), to remove undesired hybrids of low homology.

⚠ *Always prewarm the high stringency buffer to the correct temperature before adding it to the membrane. Otherwise, low homology hybrids may not be disrupted during the short washes.*

The high stringency washes recommended for the DIG System depends upon the type of hybrids on the blot, as given in the following table:

Type of Hybrid	Low Stringency Washes[1]
DNA:DNA	▶ 2×15 min in 0.5× SSC containing 0.1% SDS at 65°C (for mammalian DNA) ▶ 2×15 min in 0.1× SSC containing 0.1% SDS at 68°C (for *E. coli* DNA)
RNA:DNA or RNA:RNA	▶ 2× 15 min in 0.1x SSC containing 0.1% SDS at 68°C
Oligo:DNA	▶ At least 1× SSC containing 0.1% SDS at the temperature used for hybridization ⓘ *Optimal temperature will vary.*

[1] Always preheat the washing buffers before adding to membrane.

2

8. Detection of Probe-Target Hybrids

Probe-target hybrids are detected with an enzyme-linked immunoassay. This immunochemical detection step is more sensitive than radioactive detection procedures.

In this assay, the membrane is blocked to prevent non-specific interaction of the antibody with the filter. Alkaline phosphatase-conjugated antibody, specific for digoxigenin, recognizes the DIG molecule on the labeled hybrid.

Substrate	Development Time	Critical Points	
CDP-*Star* (chemiluminescent)	Variable *First exposure to X-ray film should be 5 – 15 min. Examine film, then make other exposures based on initial result.*	▶ No preincubation required ▶ Apply substrate quickly to avoid gray shadows (from substrate drops) and drying of membrane (leads to uneven, high background) ▶ Do not use plastic wrap to cover blot; use hybridization bags, acetate sheet protectors, or two sheets of transparency film ▶ Exposure times will be shorter than with CSPD ▶ Cannot be used with nitrocellulose ▶ Repeat exposures can be made up to two days after the addition of substrate	
CSPD (chemiluminescent)	Variable *First exposure to film1 should be 15 – 30 min. Examine film, then make other exposures based on initial result.*	▶ Activated by preincubation (10 minutes at 37°C) ▶ Apply substrate quickly to avoid drying of membrane (background) ▶ Do not use plastic wrap to cover blot; use hybridization bags, acetate sheet protectors, or two sheets of transparency film ▶ Cannot be used with nitrocellulose ▶ Repeat exposures can be made even several days after addition of substrate	
NBT/BCIP (chromogenic)[1]	5 min – overnight	▶ Must prepare dilutions fresh and store stock solutions in the dark ▶ Develop in the dark ▶ Different membranes give different colors ▶ Get only one result per experiment ▶ Stripping requires toxic dimethylformamide	

[1] For other chromogenic alkaline phosphatase (and peroxidase) substrates, see the Roche general catalog.

Addition of an alkaline phosphatase substrate allows the visualization of the hybrids. There are many different substrates available, but only two basic types of visualization assays, chromogenic or chemiluminescent. In general, chemiluminescent assays are faster (< 1 h) than chromogenic assays (overnight). However, both types of assays have advantages that make them more suitable for certain types of experiments.

To help you choose the best substrate for your application, we have listed the characteristics of several alkaline phosphatase substrates in the table below. All are available from Roche.

	Advantages	For Application, see Page

	▶ Most sensitive method
	▶ Saves time
	▶ If you are willing to use longer exposure times, you can economize on reagents:
	  ▶ Use less probe during hybridization
	  ▶ Use less antibody (1:20,000 dilution)
	  ▶ Use dilute CDP-Star (1:100, 1:200, or 1:500)
	▶ Membrane may easily be stripped and reprobed
	▶ Multiple exposures possible for each experiment
	▶ Can reuse substrate (filter, store in sodium azide)
		117 (Chapter 3)

	▶ Saves time ▶ Membrane may easily be stripped and reprobed ▶ Multiple exposures possible for each experiment ▶ Can reuse substrate (filter, store in sodium azide)	117 (Chapter 3)
	▶ Can be used with nitrocellu-lose and nylon membrane ▶ Preferred for *in situ* hybridization ▶ Shows background only from one side of membrane ▶ Signal can be renewed by rehydrating the blot	125 (Chapter 3)

2

9. Stripping and Reprobing the Membrane

After hybridization, the membrane can be stripped of DIG-labeled probe and rehybridized to a different probe. You can hybridize probes labeled with alkali-labile DIG (provided in most of our labeling kits) to a membrane, then strip the membrane with almost no loss of target sequences. The mild stripping procedures (described in the table below) allow multiple, sensitive reprobing experiments (Figure 8).

Important Tip: Membranes should never be allowed to dry before stripping. *Once dried, the membrane cannot be stripped and reprobed.*

See Section 5, page 128 in Chapter 3 for step-by-step versions of these procedures.

Brief Procedures for Stripping Alkali-Labile Probes from Blot Membranes

For This Type Blot	To Remove	Use This Procedure[1]
Southern	Chemiluminescent product and probe	▶ Rinse in H_2O, 1 min. ▶ Wash[2,3] with 0.2 M NaOH/0.1% SDS, 2× 15 min, 37°C. ▶ Rinse in 2× SSC, 5 min. Store in 2× SSC.
	Colored product (from NBT/BCIP reaction) and probe	▶ Incubate in dimethylformamide at 50° – 60°C for 1 h or more, until color has been removed. (Solution may need to be changed several times.) ▶ Rinse in H_2O, 1 min. ▶ Wash[2,3] with 0.2 M NaOH/0.1% SDS, 2× 20 min, 37°C. ▶ Rinse in 2× SSC, 5 min. Store in 2× SSC.
Northern	Chemiluminescent or colored product and probe	▶ Incubate[4,5] in 50% formamide (deionized)/5% SDS/50 mM Tris-HCl (pH 7.5), 2× 60 min, 80°C, in a sealed bag. ▶ Rinse in 2× SSC, = 5 min, RT. Store in 2× SSC[6].

[1] Membranes should never be allowed to dry before stripping. Once dried, the membrane cannot be stripped and reprobed.

[2] Researchers have successfully used this technique to probe a single Southern up to 20 times. Do not use a higher concentration of NaOH (*e.g.* 0.4 M NaOH at 65°C, as listed in some published procedures). Never use this alkali stripping procedure for Northern blots.

[3] Do not store the NaOH solution in glass containers. Use only plastic containers for storage.

[4] An alternative stripping solution for RNA probes is 90% formamide/10 mM sodium phosphate (pH 7.5). Do not use NaOH, since strong alkali will destroy the target RNA.

[5] Prepare all solutions fresh, using only RNase-free reagents.

[6] This stripping procedure allows multiple stripping and reprobing reactions. We have been able to probe a single Northern blot up to 14 times (see Figure 8).

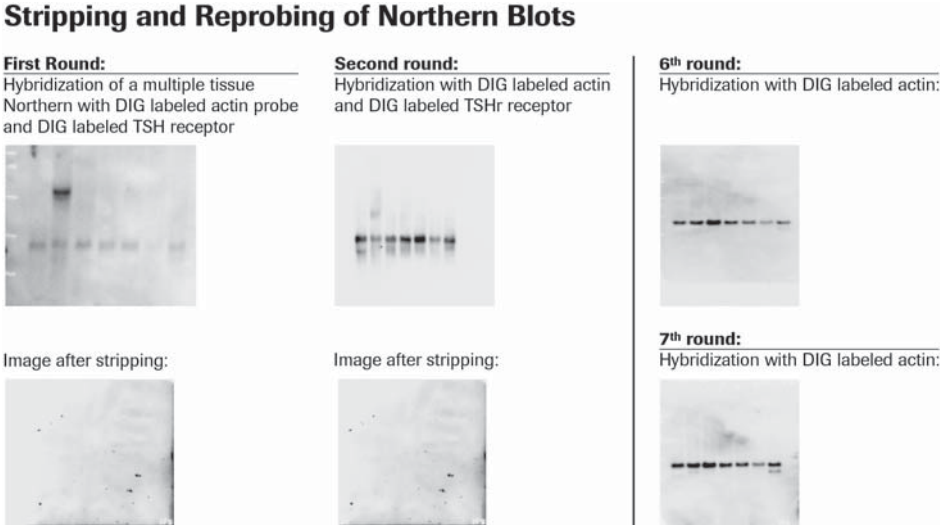


Figure 8. Stripping Alkali-labile DIG-labeled Probes from a Northern Blot for Multiple Reprobing Experiments. Two DIG-labeled RNA probes were used to analyze RNA from various tissues. One probe recognized actin RNA; the other recognized the TSH receptor (TSHr). The same membrane was hybridized repeatedly, either with both probes or the actin probe alone. Each probe was chemiluminescently detected using standard techniques (see Chapter 2). Between the experiments, the DIG-labeled probes were removed with 50% formamide/5% SDS/50 mM Tris (see table in Section 9 for details). The result from the first, second, sixth, and seventh round of hybridization are shown above.

Result: Each of the probes produced strong hybridization signals with no background. There was no substantial loss of target RNA during the stripping procedures. By using this stripping and reprobing technique, our laboratory was able to successfully analyze a single membrane up to 14 times.

2

10. Equipping a DIG Labeling and Detection Lab

The tables below list the equipment, supplies, and Roche reagents you will need to perform DIG labeling and detection experiments.

Laboratory Equipment Needed In a Typical DIG Laboratory

Equipment	Number Needed
Magnetic Stir Plate	1
Vortexer	1
Microcentrifuge	1
UV Crosslinker (*e.g.* Stratalinker)	1
Complete Horizontal Gel Electrophoresis Equipment	1 – 2
Power Supplies	1 – 2
Microwave	1
Balance	1
UV Transilluminator	1
Boiling Water Bath	1
Shaking Water Baths, adjustable (37° – 68°C)	1 – 2
PCR Thermal Cycler[1]	1
Heat Sealer, for sealing hybridization bags	1
Film Development Machine[2]	1
X-ray Film Cassettes	2 – 3
Horizontal Platform Shakers	1 – 2
Refrigerator/Freezer	1

[1] For PCR labeling only.

[2] Should be near DIG work area.

General Laboratory Supplies

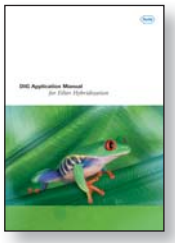
Supplies
Spatula
Erlenmeyer Flasks (200 ml, 500 ml)
Sterile Measurement Cylinders (50 ml, 100 ml, 500 ml, 1000 ml)
Glass Bottles (1 liter, 500 ml)
Magnetic Stir Bars (sterile, RNase-free)
Thermometer
Adjustable Pipettors (1 ml, 200/100 µl, 10 µl)
Sterile Tips for Pipettors
Sterile Pipettes (10 ml)
Pipetting Bulbs
Sterile Microcentrifuge Tubes (1.5 ml)
Microcentrifuge Tube Racks
Parafilm
Scissors
Forceps blunt-ended with non serrated tips
Scalpel (optional)
Whatman 3MM Paper
Gloves, Various Sizes (Powder-Free)
Permanent Marker
Sterile, Disposable 50 ml Graduated Conical Tubes
Sterile, Disposable 15 ml Graduated Conical Tubes
Laboratory Towels, Disposable
Styrofoam Microcentrifuge Tube Racks, Floating
Plastic Trays, with Lids (incubation trays for pre-hybridization, hybridization, and detection)
Petri Dishes, 150 mm (incubation trays for direct detection of labeling efficiency)
Laboratory Tissues, Disposable
Ice Buckets and Ice
Bench Paper
Aluminum Foil
Lead Ring (or some other weight) (for keeping hybridization membrane flat during incubations)
Cellulose Acetate Filters, 0.45 µm (optional, for cleaning up labeled probes)
Sterile Syringe (5 ml) (optional, for filter sterilization)
Timer
Thin-walled PCR Tubes (200 ml)
Double-distilled H_2O, filter sterilized
Double-distilled H_2O, filter sterilized, DMDC- or DEPC-treated
RNase-ZAP or similar product
Ethyl Alcohol

Reagents and Kits from Roche

Solutions/Material	Catalog Number
High Pure Nucleic Acid Purification Kits	See note 1
DNA or RNA Labeling Kit(s)	See Section 2, page 18 of this chapter
Agarose, MP	11 444 964 001
20% SDS	11 358 995 001
10× TAE buffer	11 666 690 001
DNA or RNA Molecular Weight Markers, unlabeled	See note 2
DNA or RNA Molecular Weight Markers, DIG-Labeled	See note 2
Hybridization Bags	11 666 649 001
DIG Easy Hyb buffer, ready-to-use, RNase- and DNase-free	11 603 558 001
20× SSC	11 666 681 001
Anti-DIG-alkaline phosphatase (enzyme-conjugated antibody)	11 093 274 001
DIG Wash and Block Buffer Set	11 585 762 001
Nylon Membranes, Positively Charged	11 417 240 001
Chemiluminescent substrate – Choose one:	
CDP-*Star*, ready-to-use	12 041 677 001
CSPD, ready-to-use	11 755 633 001
Recording Device:	
Lumi-Film Chemiluminescent Detection Film	11 666 916 001

11. Product Literature and Support Material

The DIG System is the nonradioactive technology of choice to label and detect nucleic acids for multiple applications such as filter hybridization or *in situ* hybridization. For an overview of our extensive DIG System product line, consult the **DIG Product Selection Guide**.



Explore detailed information about using DIG products for filter hybridization, view protocols for labeling and detection, find helpful hints and more in the **DIG Application Manual for Filter Hybridization**.

Use the **Tools for Amplification Brochure** to identify the right product for both routine and unique amplification reactions. Any amplification need and situation is addressed with this brochure.

Make use of our **PCR Application Manual**. This manual provides PCR techniques, tips, and problem-solving strategies, a convenient overview of many current PCR applications and a description of our line of PCR “tools”.

Use the comprehensive brochure **Nucleic Acid Isolation and Purification** to determine which of our products for manual purification is best for your needs.

Consult the **Nucleic Acid Isolation and Purification Manual** to see how Roche's nucleic acid preparation products can help you optimize your nucleic acid isolation results. Rely on the manual´s in-depth product description to guide you in selecting the ideal product for your application.

Tools for Mapping and Cloning
Brochure, Cat. No. 05 353 033 001.

Make use of the LAB FAQS which offers you concrete, concise answers to most frequently asked questions in modern life science research, in a practical pocket format.

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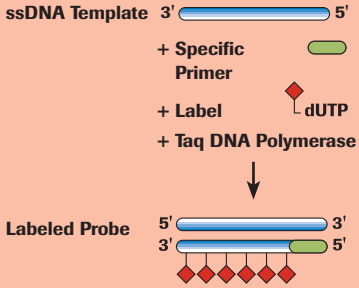
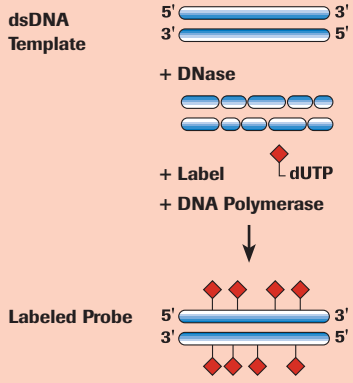
12. DIG Product Selection Guide and Ordering Information

DNA Labeling Guide

Labeling Methods		DIG - Labeling Reagents	Other Labeling Reagents
By PCR  	**Kits for Labeling**	■ **PCR DIG Probe Synthesis Kit*** 11 636 090 910 - 25 reactions ■ **PCR ELISA, DIG-Labeling** 11 636 120 910 - 50 reactions	
	Mixes for Labeling with Enzyme	■ **PCR DIG Labeling Mix*** 11 585 550 910 - 2 × 250 µl ■ **PCR DIG Labeling Mix^{PLUS}*** 11 835 289 910 - 2 × 250 µl	■ **PCR Fluorescein Labeling Mix*** 11 636 154 910 - 100 µl
	Nucleotides for Labeling	■ **Digoxigenin-11-dUTP, alkali-stable‡** 11 093 088 910 - 25 nmol (25 µl) 11 558 706 910 - 125 nmol (125 µl) 11 570 013 910 - 5 × 125 nmol (5 × 125 µl) ■ **Digoxigenin-11-dUTP, alkali-labile*** 11 573 152 910 - 25 nmol (25 µl) 11 573 179 910 - 125 nmol (125 µl) ■ **Deoxynucleoside Triphosphate Set** 11 969 064 001 - 4 × 250 µl 03 622 614 001 - 4 × 1,250 µl	■ **Biotin-16-dUTP** 11 093 070 910 - 50 nmol (50 µl) ■ **Fluorescein-12-dUTP** 11 373 242 910 - 25 nmol (25 µl) ■ **Tetramethyl-Rhodamine-5-dUTP** 11 534 378 910 - 25 nmol (25 µl)
	Enzymes	■ **Expand High Fidelity^{PLUS} PCR System¥** 03 300 242 001 - 125 U 03 300 226 001 - 500 U (2 × 250 U) 03 300 234 001 - 2,500 U (10 × 250 U)	■ **Expand High Fidelity^{PLUS} PCR System¥** 03 300 242 001 125 U 03 300 226 001 500 U (2 × 250 U) 03 300 234 001 2,500 U (10 × 250 U)
By Nick Translation  	**Kits for Labeling**		■ **Nick Translation Kit** 10 976 776 001 - 50 reactions
	Mixes for Labeling with Enzyme	■ **DIG-Nick Translation Mix*** 11 745 816 910 - 160 µl	■ **Nick Translation Mix*** 11 745 808 910 - 200 µl ■ **Biotin-Nick Translation Mix*** 11 745 824 910 - 160 µl
	Nucleotides for Labeling	■ **Digoxigenin-11-dUTP, alkali-stable‡** 11 093 088 910 - 25 nmol (25 µl) 11 558 706 910 - 125 nmol (125 µl) 11 570 013 910 - 5 × 125 nmol (5 × 125 µl) ■ **Digoxigenin-11-dUTP, alkali-labile‡** 11 573 152 910 - 25 nmol (25 µl) 11 573 179 910 - 125 nmol (125 µl) ■ **Deoxynucleoside Triphosphate Set** 11 969 064 001 - 4 × 250 µl 03 622 614 001 - 4 × 1,250 µl	■ **Biotin-16-dUTP** 11 093 070 910 - 50 nmol (50 µl) ■ **Fluorescein-12-dUTP** 11 373 242 910 - 25 nmol (25 µl) ■ **Tetramethyl-Rhodamine-5-dUTP** 11 534 378 910 - 25 nmol (25 µl)
	Enzymes	■ **DNA Polymerase I, endonuclease-free** 10 642 711 001 - 250 U 10 642 720 001 - 1,000 U ■ **DNase I recombinant, RNase-free** 04 716 728 001 - 10,000 U	■ **DNA Polymerase I, endonuclease-free** 10 642 711 001 - 250 U 10 642 720 001 - 1,000 U ■ **DNase I recombinant, RNase-free** 04 716 728 001 - 10,000 U

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Labeling Methods		DIG - Labeling Reagents	Other Labeling Reagents
By Random Priming ssDNA Template 3' — 5' + Random Primer (Hexamer) 5' — 3' + Label — dUTP + Klenow Enzyme ↓ Labeled Probe 5' — 3' / 3' — 5'	**Kits for Labeling and Detection**	■ **DIG High Prime DNA Labeling and Detection Starter Kit I*** 11 745 832 910 - 12 labeling and 24 detection reactions ■ **DIG High Prime DNA Labeling and Detection Starter Kit II*** 11 585 614 910 - 12 labeling and 24 detection reactions ■ **DIG DNA Labeling and Detection Kit*** 11 093 657 910 - 25 labeling reactions and detection of 50 blots	
	Kits for Labeling	■ **DIG DNA Labeling Kit*** 11 175 033 910 - 40 reactions	■ **Random Primed DNA Labeling Kit** 11 004 760 001 - 50 reactions
	Mixes for Labeling with Enzyme	■ **DIG-High Prime*,◆** 11 585 606 910 - 160 µl ■ **DIG DNA Labeling Mix*** 11 277 065 910 - 50 µl	■ **High Prime** 11 585 592 001 - 200 µl ■ **Biotin-High Prime◆** 11 585 649 910 - 100 µl ■ **Fluorescein-High Prime◆** 11 585 622 910 - 100 µl
	Nucleotides for Labeling	■ **Digoxigenin-11-dUTP, alkali-stable‡** 11 093 088 910 - 25 nmol (25 µl) 11 558 706 910 - 125 nmol (125 µl) 11 570 013 910 - 5 × 125 nmol (5 × 125 µl) ■ **Digoxigenin-11-dUTP, alkali-labile*** 11 573 152 910 - 25 nmol (25 µl) 11 573 179 910 - 125 nmol (125 µl)	■ **Hexanucleotide Mix** 11 277 081 001 - 100 µl ■ **Biotin-16-dUTP** 11 093 070 910 - 50 nmol (50 µl) ■ **Fluorescein-12-dUTP** 11 373 242 910 - 25 nmol (25 µl) ■ **Tetramethyl-Rhodamine-5-dUTP** 11 534 378 910 - 25 nmol (25 µl)
	Enzymes	■ **Klenow Enzyme, labeling grade** 11 008 404 001 - 100 U 11 008 412 001 - 500 U	■ **Klenow Enzyme, labeling grade** 11 008 404 001 - 100 U 11 008 412 001 - 500 U
	Additional Products	■ **Primer "random"** 11 034 731 001 - 2 mg	■ **Primer "random"** 11 034 731 001 - 2 mg

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RNA Labeling Guide

Labeling Methods		DIG - Labeling Reagents	Other Labeling Reagents
By *In Vitro* Transcription **Expression Plasmid** **Linearization** **Label + T3/T7/SP6 RNA Polymerase** UTP **Labeled Probe**	**Kits for Labeling**	■ **DIG Northern Starter Kit*** 12 039 672 910 - 10 labeling reactions and detection of 10 blots ■ **DIG RNA Labeling Kit (SP6/T7)*** 11 175 025 910 - 2 × 10 reactions	■ **SP6/T7 Transcription Kit** 10 999 644 001 - 2 × 20 reactions
	Mixes for Labeling with Enzyme	■ **DIG RNA Labeling Mix*** 11 277 073 910 - 40 µl	■ **Biotin RNA Labeling Mix** 11 685 597 910 - 40 µl ■ **Fluorescein RNA Labeling Mix** 11 685 619 910 - 40 µl
	Nucleotides for Labeling	■ **Digoxigenin-11-UTP*,‡** 11 209 256 910 - 250 nmol (10 mM, 25 µl) 03 359 247 910 - 200 nmol (3.5 mM, 57 µl)	■ **Biotin-16-UTP** 11 388 908 910 - 250 nmol (25 µl) ■ **Fluorescein-12-UTP** 11 427 857 910 - 250 nmol (25 µl) ■ **Biotin-11-CTP** 04 739 205 001 - 250 nmol (25 µl)
	Enzymes	■ **SP6 RNA Polymerase** 10 810 274 001 - 1,000 U 11 487 671 001 - 5,000 U ■ **T3 RNA Polymerase** 11 031 163 001 - 1,000 U 11 031 171 001 - 5,000 U ■ **T7 RNA Polymerase** 10 881 767 001 - 1,000 U 10 881 775 001 - 5,000 U	■ **SP6 RNA Polymerase** 10 810 274 001 - 1,000 U 11 487 671 001 - 5,000 U ■ **T3 RNA Polymerase** 11 031 163 001 - 1,000 U 11 031 171 001 - 5,000 U ■ **T7 RNA Polymerase** 10 881 767 001 - 1,000 U 10 881 775 001 - 5,000 U
	Additional Products	■ **Protector RNase Inhibitor** 03 335 399 001 - 2,000 U 03 335 402 001 - 5 × 2,000 U	■ **Protector RNase Inhibitor** 03 335 399 001 - 2,000 U 03 335 402 001 - 5 × 2,000 U

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Oligonucleotide Labeling Guide

Labeling Methods		DIG - Labeling Reagents	Other Labeling Reagents
By Tailing Oligonucleotide 5' — 3' + Label (dUTP) + Terminal Transferase Labeled Probe 5' — 3'	**Kits for Labeling**	■ **DIG Oligonucleotide 5'-End Labeling Set*** 11 480 863 001 - 10 reactions	
	Nucleotides for Labeling	■ **Digoxigenin-11-dUTP, alkali-stable‡** 11 093 088 910 - 25 nmol (25 µl) 11 558 706 910 - 125 nmol (125 µl) 11 570 013 910 - 5 × 125 nmol (5 × 125 µl) ■ **Digoxigenin-11-dUTP, alkali-labile*** 11 573 152 910 - 25 nmol (25 µl) 11 573 179 910 - 125 nmol (125 µl)	■ **Biotin-16-dUTP** 11 093 070 910 - 50 nmol (50 µl) ■ **Fluorescein-12-dUTP** 11 373 242 910 - 25 nmol (25 µl) ■ **Tetramethyl-Rhodamine-5-dUTP** 11 534 378 910 - 25 nmol (25 µl)
	Enzymes	■ **Terminal Transferase** 03 333 566 001* - 8,000 U 03 333 574 001* - 24,000 U	■ **Terminal Transferase** 03 333 566 001* - 8,000 U 03 333 574 001* - 24,000 U
By 3' -End Labeling Oligonucleotide 5' — 3' Label + Terminal Transferase (ddUTP) Labeled Probe 5' — 3'	**Kits for Labeling**	■ **DIG Oligonucleotide 3'-End Labeling Kit, 2nd generation*** 03 353 575 910 - 25 reactions ■ **DIG Gel Shift Kit, 2nd generation** 03 353 591 910 - 1 kit	
	Nucleotides for Labeling	■ **Digoxigenin-11-ddUTP** 11 363 905 910 - 25 nmol (25 µl)	■ **Biotin-16-ddUTP** 11 427 598 910 - 25 nmol (25 µl)
	Enzymes	■ **Terminal Transferase** 03 333 566 001* - 8,000 U 03 333 574 001* - 24,000 U	■ **Terminal Transferase** 03 333 566 001* - 8,000 U 03 333 574 001* - 24,000 U
By 5'-End Labeling Oligonucleotide 5' — 3' Chemical Synthesis + 5'-NH2 Group (X-NHS ester) Labeled Probe 5' — 3'	**Kits for Labeling**	■ **DIG Oligonucleotide 5'-End Labeling Set*** 11 480 863 001 - 10 reactions	
	Additional Products	■ **Digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxy-succinimide ester‡** 11 333 054 001 - 5 mg ■ **Alkaline Phosphatase** 10 713 023 001 - 1,000 U (1 U/µl) 11 097 075 001 - 1,000 U (20 U/µl)	■ **Alkaline Phosphatase** 10 713 023 001 - 1,000 U (1 U/µl) 11 097 075 001 - 1,000 U (20 U/µl)

* For general laboratory use.

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Workflow of Detection

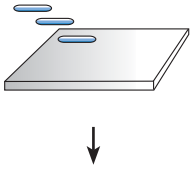
Purification of Labeled Probes

■ **High Pure PCR Product Purification Kit**	11 732 668 001 - up to 50 purifications
	11 732 676 001 - up to 250 purifications
■ **High Pure PCR Cleanup Micro Kit**	04 983 955 001 - up to 50 purifications
	04 983 912 001 - up to 200 purifications

Quick Spin Columns

■ **mini Quick Spin DNA Columns**	11 814 419 001 - 50 columns
■ **mini Quick Spin RNA Columns**	11 814 427 001 - 50 columns
■ **mini Quick Spin Oligo Columns**	11 814 397 001 - 50 columns

Immobilization of Target (Filter Bound or *In Situ*)



Agaroses

■ **Agarose LE**	11 685 660 001 – 100 g
	11 685 678 001 – 500 g
■ **Agarose MP**	11 388 983 001 – 100 g
	11 388 991 001 – 500 g
■ **Agarose MS**	11 816 586 001 – 100 g
	11 816 594 001 – 500 g

Buffers in a Box

■ **Buffers in a Box, Premixed PBS Buffer, 10x**	11 666 789 001 - 4 l
■ **Buffers in a Box, Premixed SSC Buffer, 20x**	11 666 681 001 - 4 l
■ **Buffers in a Box, Premixed TAE Buffer,**	11 666 690 001 - 4 l
■ **Buffers in a Box, Premixed TBE Buffer, 10x**	11 666 703 001 - 4 l

Nucleic Acids and Probes, DIG-labeled

■ **DIG-labeled Control DNA***	11 585 738 910 - 50 µl
■ **DIG-labeled Control RNA***	11 585 746 910 - 50 µl
■ **Actin RNA Probe, DIG-labeled**	11 498 045 001 - 2 µg

DNA Molecular Weight Markers, DIG-labeled

■ **DNA Molecular Weight Marker II, DIG-labeled***	11 218 590 910 - 5 µg (500 µl)
■ **DNA Molecular Weight Marker III, DIG-labeled***	11 218 603 910 - 5 µg (500 µl)
■ **DNA Molecular Weight Marker VI, DIG-labeled***	11 218 611 910 - 5 µg (500 µl)
■ **DNA Molecular Weight Marker VII, DIG-labeled***	11 669 940 910 - 5 µg (500 µl)
■ **DNA Molecular Weight Marker VIII, DIG-labeled***	11 449 451 910 - 5 µg (500 µl)

RNA Molecular Weight Markers, DIG-labeled

■ **RNA Molecular Weight Marker I, DIG-labeled***	11 526 529 910 - 4 µg (200 µl)
■ **RNA Molecular Weight Marker II, DIG-labeled***	11 526 537 910 - 2 µg (200 µl)
■ **RNA Molecular Weight Marker III, DIG-labeled***	11 373 099 910 - 2 µg (200 µl)

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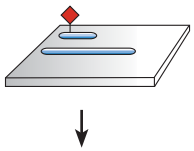
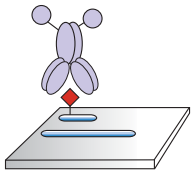
Hybridization of Labeled Probes



■ **Blocking Reagent**	11 096 176 001 – 50 g
■ **DIG Easy Hyb**	11 603 558 001 – 500 ml
■ **DIG Wash and Block Buffer Set**	11 585 762 001 - 1 set (30 blots)
■ **Nylon Membranes, positively charged**	11 209 272 001 - 10 sheets (20 × 30 cm) 11 209 299 001 - 20 sheets (10 × 15 cm) 11 417 240 001 - 1 roll (0.3 × 3 m)
■ **Nylon Membranes for Colony and Plaque Hybridization**	11 699 075 001 - 50 discs (each 82 mm diameter) 11 699 083 001 - 50 discs (each 132 mm diameter)
■ **Lumi-Film Chemiluminescent Detection Film**	11 666 916 001 - 100 films (7.1 × 9.4 inches ≅ 18 × 24 cm)
■ **Hybridization Bags**	11 666 649 001 - 50 bags
■ **Buffers in a Box**	see page 6

Detection – Binding of Antibody or Antibody Conjugate to Labeled Probe



Available Antibodies and their Recommended Use

Filter Hybridization	*In situ* Hybridization	Tube and Microplate Formats (ELISA)
With Conjugation		
Anti-Digoxigenin-AP, Fab fragments*	**Anti-Digoxigenin-AP, Fab fragments***	**Anti-Digoxigenin-AP, Fab fragments***
11 093 274 910 - 150 U (200 µl)	11 093 274 910 - 150 U (200 µl)	11 093 274 910 - 150 U (200 µl)
Anti-Digoxigenin-POD, Fab fragments*	**Anti-Digoxigenin-Fluorescein, Fab fragments***	**Anti-Digoxigenin-POD, Fab fragments***
11 207 733 910 – 150 U	11 207 741 910 - 200 µg	11 207 733 910 – 150 U
Anti-Fluorescein-AP, Fab fragments	**Anti-Digoxigenin-POD, Fab fragments***	**Anti-Fluorescein-AP, Fab fragments**
11 426 338 910 - 150 U (200 µl)	11 207 733 910 – 150 U	11 426 338 910 - 150 U (200 µl)
Anti-Fluorescein-POD, Fab fragments	**Anti-Digoxigenin-Rhodamine, Fab fragments***	**Anti-Fluorescein-POD, Fab fragments**
11 426 346 910 – 150 U	11 207 750 910 - 200 µg	11 426 346 910 – 150 U
	Anti-Fluorescein-AP, Fab fragments	**Streptavidin-AP, for biotin**
	11 426 338 910 - 150 U (200 µl)	11 093 266 910 -150 U
	Streptavidin-AP, for biotin	**Streptavidin-POD, for biotin**
	11 093 266 910 -150 U	11 089 153 001 – 500 U
	Streptavidin-POD, for biotin	
	11 089 153 001 – 500 U	
Without Conjugation		
Anti-Digoxigenin, polyclonal from sheep*	**Anti-Digoxigenin, polyclonal from sheep***	**Anti-Digoxigenin, polyclonal from sheep***
11 333 089 001 - 200 µg	11 333 089 001 - 200 µg	11 333 089 001 - 200 µg
Anti-Digoxigenin, monoclonal*	**Anti-Digoxigenin, monoclonal***	**Anti-Digoxigenin, monoclonal***
11 333 062 910 - 100 µg	11 333 062 910 - 100 µg	11 333 062 910 - 100 µg
	Anti-Digoxigenin, Fab fragments*	**Anti-Digoxigenin, Fab fragments***
	11 214 667 001 – 1 mg	11 214 667 001 – 1 mg
	Anti-Fluorescein, monoclonal	**Anti-Fluorescein, monoclonal**
	11 426 320 001 - 100 µg	11 426 320 001 - 100 µg

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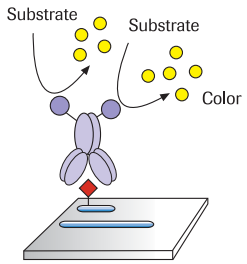
Detection using Alkaline Phosphatase (AP)

		Filter Hybridization	*In Situ* Hybridization	Tube and Microplate Formats (ELISA)
		Colorimetric Detection – Alkaline Phosphatase (AP)		
	**Conjugated Antibodies**	■ **Anti-Fluorescein-AP, Fab fragments** 11 426 338 910 - 150 U (200 µl) ■ **Anti-Digoxigenin-AP, Fab fragments*** 11 093 274 910 - 150 U (200 µl)	■ **Streptavidin-AP, for biotin** 11 093 266 910 - 150 U ■ **Anti-Fluorescein-AP, Fab fragments** 11 426 338 910 - 150 U (200 µl) ■ **Anti-Digoxigenin-AP, Fab fragments*** 11 093 274 910 - 150 U (200 µl)	■ **Streptavidin-AP, for biotin** 11 093 266 910 - 150 U ■ **Anti-Fluorescein-AP, Fab fragments** 11 426 338 910 - 150 U (200 µl) ■ **Anti-Digoxigenin-AP, Fab fragments*** 11 093 274 910 - 150 U (200 µl)
	Single Reagents	■ **BM purple, AP substrate precipitating** 11 442 074 001 – 100 ml ■ **NBT/BCIP Ready-to-Use Tablets** 11 697 471 001 – 20 tablets ■ **NBT/BCIP Stock Solution** 11 681 451 001 – 8 ml	■ **BM purple, AP substrate precipitating** 11 442 074 001 – 100 ml ■ **NBT/BCIP Ready-to-Use Tablets** 11 697 471 001 – 20 tablets ■ **NBT/BCIP Stock Solution** 11 681 451 001 – 8 ml ■ **Fast Red Tablets** 11 496 549 001 – 20 tablets	
	Kits	■ **DIG-High Prime DNA Labeling and Detection Starter Kit I*** 11 745 832 910 - 12 labeling and 24 detection reactions ■ **DIG DNA Labeling and Detection Kit*** 11 093 657 910 - 25 labeling reactions and 50 blots		

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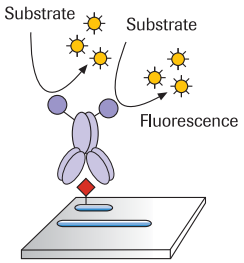
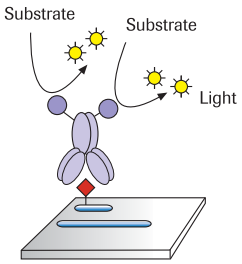
		Filter Hybridization	*In Situ* Hybridization	Tube and Microplate Formats (ELISA)
Fluorescent Detection – Alkaline Phosphatase (AP)				
	**Single Reagents**			■ **AttoPhos** 11 681 982 001 – for 1,800 wells or 720 tubes
	Kits	■ **HNPP Fluorescent Detection Set** 11 758 888 001 – 1 set	■ **HNPP Fluorescent Detection Set** 11 758 888 001 – 1 set	
	**Conjugated Antibodies**	■ **Anti-Fluorescein-AP, Fab fragments** 11 426 338 910 - 150 U (200 µl) ■ **Anti-Digoxigenin-AP, Fab fragments*** 11 093 274 910 - 150 U (200 µl)	■ **Streptavidin-AP, for biotin** 11 093 266 910 - 150 U ■ **Anti-Fluorescein-AP, Fab fragments** 11 426 338 910 - 150 U (200 µl) ■ **Anti-Digoxigenin-AP, Fab fragments*** 11 093 274 910 - 150 U (200 µl)	■ **Streptavidin-AP, for biotin** 11 093 266 910 - 150 U ■ **Anti-Fluorescein-AP, Fab fragments** 11 426 338 910 - 150 U (200 µl) ■ **Anti-Digoxigenin-AP, Fab fragments*** 11 093 274 910 - 150 U (200 µl)
	Single Reagents	■ **CSPD, ready-to-use** 11 755 633 001 – 2 × 50 ml ■ **CDP-*Star*, ready-to-use** 12 041 677 001 – 2 × 50 ml		
	Kits	■ **DIG Northern Starter Kit*** 12 039 672 910 - 10 labeling reactions and detection of 10 blots 10 × 10 cm^2) ■ **DIG-High Prime DNA Labeling and Detection Starter Kit II*** 11 585 614 910 - 12 labeling and 24 detection reactions ■ **DIG Luminescent Detection Kit*** 11 363 514 910 – 50 blots		■ **BM Chemiluminescence ELISA Substrate (AP)** 11 759 779 001 – 150 ml

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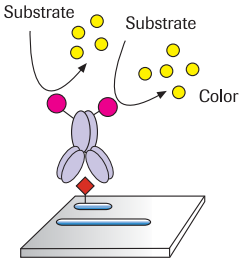
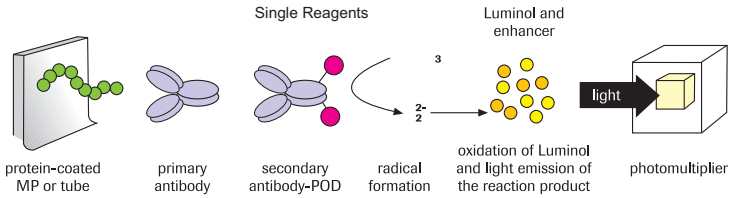
Detection using Peroxidase (POD)

		Filter Hybridization	*In Situ* Hybridization	Tube and Microplate Formats (ELISA)
Colorimetric Detection – Peroxidase (POD)				
	**Antibodies**	■ **Anti-Fluorescein-POD, Fab fragments** 11 426 346 910 – 150 U ■ **Anti-Digoxigenin-POD, Fab fragments*** 11 207 733 910 – 150 U	■ **Streptavidin-POD, for biotin** 11 089 153 001 – 500 U ■ **Anti-Digoxigenin-POD, Fab fragments*** 11 207 733 910 – 150 U	■ **Streptavidin-POD, for biotin** 11 089 153 001 – 500 U ■ **Anti-Fluorescein-POD, Fab fragments** 11 426 346 910 – 150 U ■ **Anti-Digoxigenin-POD, Fab fragments*** 11 207 733 910 – 150 U
	Single Reagents	■ **BM Blue POD Substrate, precipitating, TMB ready-to-use solution** 11 442 066 001 – 100 ml ■ **DAB Substrate, metal enhanced, precipitating** 11 718 096 001 – 1 pack	■ **DAB Substrate, metal enhanced, precipitating** 11 718 096 001 – 1 pack	■ **ABTS Solution, ready-to-use** 11 684 302 001 – 3 × 100 ml ■ **ABTS Tablets** 11 204 521 001 – 20 tablets ■ **BM Blue POD Substrate, soluble** 11 484 281 001 – 100 ml
	Kits			■ **PCR ELISA, DIG Detection** 11 636 111 910 – 192 reactions ■ **PCR ELISA, DIG Detection, 5-pack** 11 965 409 910 – 1 kit
Chemiluminescent Detection –Peroxidase (POD)				
				■ **BM Chemiluminescence ELISA Substrate (POD)** 11 582 950 001 – 250 ml

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3

Procedures for Nonradioactive Labeling and Detection

3

1. Introduction

This chapter summarizes all the information you need to perform the most commonly used methods for DIG labeling and detection. This information was gathered during years of work with the DIG system, both in our laboratories and in the laboratories of our customers. It is a true "insider's guide" to getting the most out of the DIG system.

In addition to basic protocols for both labeling and detection, this chapter will tell you:

- All the Roche products and other materials you need for the protocols
- Tips, troubleshooting guides, and/or additional information for getting the best DIG results
- Typical results obtained with the DIG System, and
- High volume screening applications for the DIG System

Read this chapter to familiarize yourself with the basic methods. If you need information about variations on any basic method, you can consult the package inserts included with the DIG products listed in this chapter.

Tip: For an overview of how to use the procedures in this chapter, see "An Overview of the DIG Application Manual," page 12.

The methods described in this chapter include:

2. Techniques for DIG Labeling of Hybridization Probes

The DIG system offers many ways to label a hybridization probe with digoxigenin (DIG). This section discusses five of them. The table below will help you decide which labeling method is best for your purposes:

Advantages of Different DIG Labeling Techniques

Method	Probe	Advantages
Random Primed Labeling (High Yield Method)	**DNA**	▶ Random primed labeling inserts one DIG moiety in every stretch of 20 – 25 nucleotides. This ratio allows probes to detect as little as 0.1 pg target DNA. ▶ Compared with the standard random primed labeling mix (found in the original DIG DNA Labeling Kit), DIG-High Prime generates a significantly higher yield of newly synthesized DNA in less time. ▶ Random primed labeling reactions can be scaled up or down at least tenfold, allowing production of labeled probe from either very small (10 ng) or very large (up to 10 µg) amounts of template.
PCR Labeling	**DNA**	▶ Only a small amount of template DNA is required (no more than 10 pg plasmid DNA or 1 – 50 ng genomic DNA). ▶ Purity of template is not as critical for PCR labeling as for other types of labeling (especially random primed labeling). ▶ Labeling reaction requires less optimization than other methods [if Expand High Fidelity enzyme blend (included in PCR DIG Probe Synthesis Kit) is used]. ▶ Yield of labeled probe is very high. ▶ Evaluation of labeling efficiency may be done very quickly by gel electrophoresis.
Transcriptional Labeling	**RNA**	▶ DIG-labeled RNA probes offer much better sensitivity for detecting RNA targets than DNA probes (see Figure 13, page 83). In addition, the RNA probes generate a higher signal-to-noise ratio on Northern blots. ▶ DIG-labeled RNA probes can detect rare mRNAs in small amounts (=1 µg; in some cases as little as 0.1 µg) of total human or plant RNA. ▶ Large amounts of labeled probe can be generated from a single reaction (*e.g.*, 10 µg RNA from 1 µg DNA template). ▶ Labeling reaction can easily be scaled up to produce milligrams of product. ▶ RNA probes are single-stranded and cannot re-anneal to an opposite strand. ▶ DIG-labeled RNA probes can easily be fragmented by mild alkaline treatment for use in *in situ* hybridization.
DIG Oligo Labeling	**Oligo-nucle-otide**	▶ Small probe hybridizes to target faster, so hybridization times are reduced. ▶ Oligonucleotide probes are single-stranded, so they cannot self-hybridize (renature) during the hybridization reaction. ▶ Oligonucleotide sequences can be custom synthesized, so target recognition is optimized. ▶ Oligonucleotides are small and diffuse readily into cells, making them ideal for *in situ* hybridization applications.

Once you have decided the best labeling method for your probe, use the table below to find the detailed labeling procedure.

For information on this topic	Turn to Section	Starting on page
Random Primed Labeling of DNA Probes (High Yield Method) **Key Product:** DIG-High Prime or DIG-High Prime DNA Labeling and Detection Starter Kit II	2.1	57
PCR Labeling of DNA Probes **Key Product:** PCR DIG Probe Synthesis Kit	2.2	65
Transcriptional Labeling of RNA Probes **Key Product:** DIG Northern Starter Kit	2.3	74
DIG Oligonucleotide Labeling **Key Products:** Oligonucleotide 3' End Labeling and Tailing Kits, 2nd generation	2.4	84
Estimation of Probe Yield by the Direct Detection Procedure	2.5	85

2.1 Random Primed Labeling of DNA Probes (High Yield Method)

Random primed labeling is the most widely used method for generating homogeneously labeled DNA probes. It produces sensitive probes that can detect single-copy genes even in complex tar-gets (such as human and plant genomic DNA).

In random primed labeling, template DNA is first linearized and denatured. Then, Klenow polymerase incorporates DIG-dUTP into the template as multiple locations that are determined by the binding of a random hexamer primer mixture.

Now it is possible to get higher yields from random primed labeling by using DIG-High Prime. DIG-High Prime is a convenient, optimized version of the standard (original) random primed labeling mix. Compared to the standard method, DIG-High Prime generates a significantly higher yield of newly synthesized DNA in less time, starting from fewer templates.

Example: *In general, DIG-High Prime can produce as much newly synthesized DNA in one hour as the standard random primed labeling mix can produce in an overnight incubation:*

	Amount of Newly Synthesized DNA (ng) Produced			
	with DIG-High Prime		with Standard Labeling Mix	
Amount of Purified Template (ng)	in 1 h	in 20 h	in 1 h	in 20 h
300	450	2000	120	500
1000	850	2300	260	780
3000	1350	2650	530	890

This section describes how to use DIG-High Prime to prepare DIG-labeled DNA probes. Topics in this section include:

3

2.1.1 Materials Required for Random Primed Labeling

RAS Products Required for Random Primed Labeling

Product	Catalog Number	Description
DIG-High Prime	11 585 606 910	▶ Premixed, 5× conc. solution for random primed labeling reaction
DIG-High Prime DNA Labeling and Detection Starter Kit II (all-in-one kit, containing DIG-High Prime [vial 1], plus reagents needed for estimation of probe yield [vials 2, 3], hybridization of probe to a Southern blot [vial 7], and chemiluminescent detection of probe-target hybrids [vials 4–6])	11 585 614 910	▶ DIG-High Prime labeling mix, 5× conc. (vial 1)[1] ▶ DIG-labeled Control DNA (vial 2)[1] ▶ DNA Dilution buffer (vial 3)[1] ▶ Anti-DIG-alkaline phosphatase conjugate (vial 4)[1] ▶ CSPD chemiluminescent substrate, ready-to-use (vial 5)[1] ▶ Blocking solution, 10x conc. (vial 6)[1] ▶ DIG Easy Hyb granules (vial 7)[1]

[1] These kit components are also available as separate reagents. See ordering information in Chapter 2 (starting page 44).

Additional Materials Required for Protocol

Reagent/Equipment	Description/Purpose
Template DNA	▶ **Use highly purified** DNA fragments of at least 100 bp or linearized plasmid/cosmid/lambda DNA, containing the sequence to be labeled. *The procedure given in this section can be used to label 10 ng – 3 µg plasmid DNA. However, be sure to use enough template to generate the amount of probe you need. For example, use at least 300 ng template if you want the probe to detect single-copy genes in complex genomes.*
Autoclaved, double dist. H_2O	For diluting template DNA
Stop buffer	EDTA, 0.2 M, pH 8.0, sterile
Ice/water bath	For denaturing DNA
Water bath(s)	Temperatures required: ▶ Boiling water, for denaturing DNA ▶ 37°C, for labeling reaction ▶ 65°C, for stopping reaction

2.1.2 Procedures

The random primed labeling protocol involves the procedures listed in the flow chart below. An estimate of the time required for each procedure is also given in the chart.

break *This icon means you can stop the protocol at the end of the procedure and store the product for a time before starting the next procedure.*

2.1.2.1. Purification of Template

For best results, we recommend careful preparation and purification of all templates. Ideally, cloned inserts, isolated free of vector sequences, should be used as template in the labeling reaction. This eliminates the possibility that vector sequences will be labeled and lead to unwanted cross-hybridization with "vector-like" sequences in the target DNA.

The table below lists the different possible purification methods for preparing highly purified random primed labeling templates (plasmid DNA).

Purification Method	Comments
Centrifuge in a caesium chloride density gradient	▶ This is the slowest, but best method for preparing highly purified template. ▶ Method may require overnight centrifugation. ▶ After purification, digest DNA with a restriction enzyme to excise the cloned target sequence from the vector sequences[1].
Use the High Pure Plasmid Isolation Kit (Cat. No. 11 754 777 001)	▶ Preferred alternative if density gradient purification is not possible. ▶ Requires only 30 min. ▶ Templates prepared with the kit may be used directly in the labeling reaction. ▶ Eliminates *E. coli* DNA from the purified product. ▶ After purification, digest DNA with a restriction enzyme to excise the cloned target sequence from the vector sequences[1].
Excise DNA from an agarose gel	▶ For best results, use either our High Pure PCR Product Purification Kit (Cat. No. 11 732 668 001 or Cat. No. 11 732 676 001) or our Agarose Gel DNA Extraction Kit (Cat. No. 11 696 505 001) to separate the DNA from the agarose[1]. ▶ Elutips, from Schleicher and Schuell, offer an alternative for separating the DNA from the agarose.
Use a commercially available kit from another vendor	▶ If you are preparing plasmid from *E. coli*, choose a kit that does not contaminate the purified plasmid with *E. coli* chromosomal DNA. (!) *The presence of E. coli chromosomal sequences in the template will cause specific (due to homologies with repetitive sequences), but undesired signals in subsequent hybridization experiments.* ***If this occurs, use alternative labeling method (e.g. PCR) to label probes.*** ▶ After using the kit, perform a final phenol/chloroform extraction on the purified DNA to remove residual proteins that may inhibit the labeling reaction. ▶ After organic extraction, precipitate the extracted DNA with ethanol and resuspend it in low concentrations of Tris (*e.g.*, 10 mM Tris, pH 7.5) or repurified water. Avoid high EDTA concentrations (as in TE buffer), since these can inhibit the enzymatic labeling reaction. ▶ After purification, digest DNA with a restriction enzyme to excise the cloned target sequence from the vector sequences[1].
Use a crude plasmid preparation method (mini-preps, maxi-preps)	▶ This is the quickest method, but is likely to give less than optimal results. ▶ After purification, digest DNA with a restriction enzyme to excise the cloned target sequence from the vector sequences[1]. ▶ If you use a crude plasmid preparation as template in the labeling reaction, you should purify the labeled probe with our High Pure PCR Product Purification Kit (Cat. No. 11 732 668 001 or 11 732 676 001) before using it in a hybridization experiment. (See Section 2.1.3.3, "Cleaning up Dirty Labeled Probes to Remove Background," on page 63 in this chapter).

[1] You may also use supercoiled, circular plasmid DNA in the reaction, but linearized DNA will usually give better results. If your template DNA is >5 kb, digest it with a restriction enzyme that has a 4 bp recognition sequence (such as Hae III).

3

2.1.2.2. Random Primed Labeling of Probe

Before you start: Be sure you have enough highly purified template to generate the amount of DIG-labeled probe you need (see yield table in this section). Generally, for single-copy gene detection on a blot, you will need 25 ng labeled probe per ml of hybridization solution. We recommend to start with at least 300 ng of template.

Use the following procedure to label 10 ng to 3 µg of purified template DNA.

Step	Action	Time
1	Prepare the sample as follows: ▶ Add 10 ng - 3 µg template DNA (linear or supercoiled) to a reaction vial. ***Tip:*** Use at least 300 ng template if you want the probe to detect single-copy genes in complex genomes. ▶ Add autoclaved, double dest. water to a final volume of 16 µl.	1 min
2	Denature the DNA as follows: ▶ Heat the sample in a boiling water bath for 10 min. ▶ Quickly chill the sample in an ice/water bath.	10 min
3	Label the DNA as follows: ▶ Mix DIG-High Prime thoroughly. ▶ Add 4 µl mixed DIG-High Prime to the denatured sample. ▶ Centrifuge briefly. ▶ Incubate sample for at least 1 h at 37°C. ***Tip:*** If you have a limited amount of template, you may continue the incubation overnight (up to 20 h) to increase the yield of labeled probe.	1 h overnight
4	Stop the reaction as follows: ▶ Add 2 µl 0.2 M EDTA (pH 8.0) to the sample, and/or ▶ Heat the sample to 65°C for 10 min.	10 min

2.1.2.3. Determination of Probe Yield

No hybridization can be successful unless an optimal amount of probe is used. Quantification of the labeled probe is the first step in determining the optimal amount of probe. Therefore, after completion of the labeling reaction, the next step is determining how much labeled probe you made. This determination must be done before the probe can be used successfully in a hybridization reaction.

To learn how to do estimate the yield of your labeled probe, go to Section 2.5, "Estimation of Probe Yield by the Direct Detection Procedure" on page 85 of this chapter.

To assist you in estimating the amount of labeled probe, the table below lists typical yields for optimized random primed labeling reactions. These are estimates only and do not necessarily reflect the amount of labeled probe produced in your labeling reaction.

Yields for Random Primed Labeling with the DIG-High Prime Labeling Mix

	Amount of Newly Synthesized DNA[1] (ng) Produced in	
Amount of Purified Template (ng)	**1 h**	**20 h**
10	45	600
30	130	1050
100	270	1500
300	450	2000
1000	850	2300
3000	1350	2650

[1] The yield table above shows the new DNA expected from the 20 µl labeling reaction under optimal conditions. For tips on increasing the yield of the reaction, see Section 2.1.3.1 below.

2.1.2.4. What To Do Next

IF you want to...	THEN...
See typical results achieved with random primed labeled probes	See "How to Optimize Single Copy Gene Detection Easily" on page 176 in chapter 5 B.
Use your labeled probe to detect DNA on a Southern or dot blot	Go to Section 3.1, "Hybridization of DNA Probes to a Southern Blot," on page 94 of this chapter.
Use you labeled probe to detect recombinant clones in a bacterial or phage library	Go to Section 6.1, "Use of DIG-labeled Probes for Colony and Plaque Hybridization," on page 131 of this chapter.
Store your labeled probe for later use	Store the labeled probe at –20°C. **Tip:** Labeled probes are stable for at least 1 year when they are stored at –20°C. In most cases, labeled probes are stable for considerably longer (often as long as 4 years).

3

2.1.3 Getting the Best Results from Random Primed Labeling

2.1.3.1. Critical Hints about Random Primed Labeling

Factor	Hints
Template purification	▶ Labeling efficiency depends greatly on the purity of the DNA template. If a labeling reaction fails, it is most likely due to impure template. ▶ For best results, template DNA must be linearized and denatured prior to labeling. Ideally, cloned inserts, isolated free of vector sequences, should be used as template in the labeling reaction.
Template amount	▶ Be sure to use enough template or run the reaction long enough to generate the amount of probe you need (see yield table in Procedure 2.1.2.2). ▶ Labeling efficiency for a given amount of template can be drastically increased by either of the following methods: ▶ Label the probe for a longer time. If you have only a limited amount of template, label the probe overnight (up to 20 h). ▶ Scale up the labeling reaction to 200 –300 µl (but do not increase the amount of template). *When the reaction is scaled up, the amount of newly synthesized DNA can actually exceed the amount of template DNA in the reaction, effectively amplifying the amount of probe produced. The exact mechanism behind this phenomenon is unknown, but is most likely due to strand displacement by the Klenow enzyme, which allows more rapid copying of the template.*
Labeled probe	▶ The random primed method can label even very short DNA probes. However, for best results when labeling very short probes, use the PCR labeling method (see Section 2.2 in this chapter) ▶ A probe prepared by random primed labeling will not be a single DNA species, but a collection of fragments that vary in length. That means: ▶ Random primed probes will be shorter than the full-length template DNA. ***Example**: Labeled fragments from a 2 kb probe will be 300 to 1500 bp, with most about 800 bp.* ▶ The labeled probe will appear as a "smear", rather than a unique band on an electrophoretic gel.

2.1.3.2. Troubleshooting the Random Primed Labeling Procedures

Problem	Possible cause	Recommendation
Low probe labeling efficiency	Labeling conditions not optimal	▶ Repeat labeling reaction, but incubate for a longer time (up to overnight). ▶ Leave the amount of template the same, but increase the reaction volume; scale up all other components of the reaction mixture proportionately. ▶ Denature template in a boiling water bath.
	Impure template	▶ Use only highly purified template. ▶ Follow the guidelines in Procedure 2.1.2.1 for preparing template. ▶ Purify template DNA by phenol/chloroform extraction and ethanol precipitation before labeling.
High background during chemiluminescent detection (Section 4.1 in this chapter)	Inefficient labeling	▶ See above comments under "Low probe labeling efficiency."
	Other causes	▶ See "Troubleshooting the Hybridization Blot," Section 3.3.2 on page 114 of this chapter.

2.1.3.3. Cleaning Up "Dirty" Labeled Probes to Remove Background

Occasionally, you may want to use crude plasmid preparations as template in the random primed labeling reaction. Our experiments indicate that labeled probes purified from such crude preparations can be used **if** they are first purified with the High Pure PCR Product Purification Kit (Cat. No. 11 732 668 001 or 11 732 676 001). We have found that the kit is ideal for removing components of "dirty" random prime-labeled probe preparations that can cause high background (as demonstrated in Figure 9).

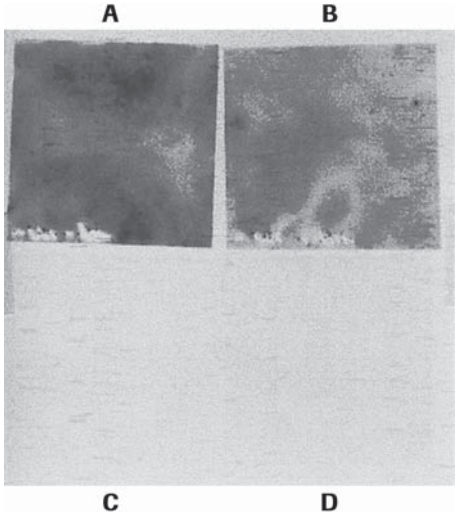


Figure 9. Removal of Strong Background from Random Prime Labeled Probes with the High Pure PCR Product Purification Kit. A very "dirty" (*i.e.*, containing proteins) plasmid preparation (prepared by the boiling method, without phenol extraction) was used as template for random primed labeling. To test for non-specific background, the labeled probe was applied in a mock hybridization (panel A) to a blot membrane containing no target. The hybridization solution contained 25 ng labeled probe per ml DIG Easy Hyb buffer. Standard stringent washes were used to remove unbound probe. Probe remaining on the washed membrane was detected and visualized immunochemically. The mock hybridization was repeated with probe that had been filtered through cellulose acetate (panel B), probe that had been purified with the High Pure Product Purification Kit (panel C), and probe that had been purified with the High Pure Kit, then filtered (panel D).

Results: When used directly (without purification), the probe produced high background on the blot membrane (panel A). Even after the probe was filtered through a 0.45 µm sterile cellulose acetate filter, the probe still produced high background (panel B). However, after the probe was cleaned up with the High Pure PCR Product Purification Kit, the purified probe produced very little background (panels C, D).

Conclusion: The High Pure Product Purification Kit (purification time, 10 min) removed the background-causing components of a dirty random prime-labeled probe (panel C). No further treatment (*e.g.* with cellulose acetate filtration, panel D) was necessary.

3

2.2 PCR Labeling of DNA Probes

PCR labeling is rapidly becoming the preferred method for preparing DIG-labeled probes when the template is available in only limited amounts, is only partially purified, or is very short. It requires less optimization than other methods and produces a high yield of labeled probe.

In PCR labeling, a thermostable polymerase incorporates DIG-dUTP as it amplifies a specific region of the template DNA. The result is a highly labeled, very specific, and very sensitive hybridization probe.

This section describes how to use the PCR DIG Probe Synthesis Kit to label DNA probes. Topics in this section include:

2.2.1 Materials required for PCR Labeling

Key Product Required for PCR Labeling

Product	Catalog Number	Reagents Included or Description
PCR DIG Probe Synthesis Kit	1 1 636 090 910	▶ Enzyme mix, Expand High Fidelity (vial 1) ▶ PCR DIG probe synthesis mix, 10× conc. (vial 2) ▶ PCR buffer with $MgCl_2$, 10× conc. (vial 3) ▶ dNTP stock solution, 10× conc. (vial 4) ▶ Control template, human tPA (vial 5) ▶ Control PCR primer mix, human tPA (vial 6)

Additional Materials Required for Protocol

Reagent	Description
Template DNA	Partially purified DNA containing the sequence to be labeled. Use either: ▶ Plasmid DNA, 10 – 100 pg (optimal amount, 10 pg) ▶ Genomic DNA, 1 – 50 ng (optimal amount, 10 ng) *Purity of template is not as critical for PCR labeling as for other types of labeling. For example, suitable templates include partially purified plasmid prepared by any of a variety of "quick preparation" methods. However, for best results, use a rapid DNA purification kit, such as our High Pure PCR Template Preparation Kit (Cat. No. 11 796 828 001) to prepare your template.*
PCR primers	Primers that amplify the sequence to be labeled. You will need: ▶ 0.1 – 1 µM solution of upstream primer ▶ 0.1 – 1 µM solution of downstream primer
Mineral oil	▶ For overlaying amplification reactions to prevent evaporation (not needed in some thermal cyclers)
Reagents for agarose mini-gel	▶ For checking the synthesis of the labeled probe

2.2.2 Procedures

The PCR labeling protocol involves the procedures listed in the flow chart below. An estimate of the time required for each procedure is also given in the chart.

2.2.2.1 (page 66)	**PCR Labeling of Probe** Stage A: Prepare template Stage B: Optimize PCR parameters for each template-primer set Stage C: Amplify template in the presence of DIG-dUTP	20 – 30 min Variable approx. 2 h
2.2.2.2 (page 69)	**Checking the Synthesis of Labeled Probe**	approx. 1 h

This icon means you can stop the protocol at the end of the procedure and store the product for a time before starting the next procedure.

3

2.2.2.1. PCR Labeling of Probe

Use the following procedure to label 10 ng genomic DNA or 10 pg plasmid DNA. The reaction can produce enough labeled probe to analyze 650 cm^2 of blot membrane.

The numbered vials refer to components of the PCR DIG Probe Synthesis Kit.

Step	Action	Time
1	Depending on the source of the target, prepare template DNA by any of the following methods: **IF you are isolating...** → **THEN prepare template with...** Genomic DNA from blood, buffy coat, cultured cells, tissue, mouse tail, yeast, bacteria, or paraffin-embedded tissue sections → High Pure PCR Template Preparation Kit[1] Plasmid DNA from bacterial cultures → ▶ High Pure Plasmid Isolation Kit[1], or ▶ Any of a variety of "quick preparation" methods, even simple boiling of cells Viral DNA → High Pure Viral Nucleic Acid Kit[1] [1] For ordering information, see page 44 of Chapter 2.	20 – 30 min
2	To optimize the amplification of your target, use the target DNA you isolated and the PCR primers you designed to perform a series of "test PCRs" (*e.g.*, with different concentrations of Mg^{2+} and different thermal cycler programs). (see Step 6 for suggested amplification conditions to use in initial experiments.) *For PCR optimization guidelines and procedure, see Chapter 2 of the latest edition of the PCR Applications Manual.*	Variable

▶▶▶

Step	Action	Time

3 For each experimental or control sample, add the following components to a sterile microcentrifuge tube. Place the tube on ice during pipetting. — 5 min

Reagent	DIG-labeled experimental probe[1]	Unlabeled control probe[2]	DIG-labeled tPA control probe[3]	Final Concentration
Sterile, repurified H_2O	variable volume	variable volume	29.25 µl	–
PCR buffer with $MgCl_2$, 10× conc. (vial 3)	5 µl	5 µl	5 µl	1×[4]
PCR DIG mix, 10× conc. (vial 2)	5 µl	-	5 µl	200 µM dNTPs
dNTP stock solution, 10× conc. (vial 4)[5]	-	5 µl	-	200 µM dNTPs
Upstream and downstream primers	Variable volume	Variable volume	5 µl (vial 6)	0.1 – 1 µM each primer
Enzyme mix, Expand High Fidelity (vial 1)	0.75 µl	0.75 µl	0.75 µl	2.6 units total enzyme
Template DNA	Variable volume	Variable volume	5 µl (vial 5)	10 ng genomic DNA, or 10 pg plasmid DNA
Total reaction volume	50 µl	50 µl	50 µl	

[1] Experimental sample to produce labeled DNA for use as a hybridization probe.

[2] Unlabeled positive control, identical to experimental sample except the reaction 2 mix contains no DIG-dUTP.

This control is required for evaluating probe labeling (Procedure 2.2.2.2).

[3] Labeled positive control; produces a labeled probe that recognizes human tissue plasminogen activator (tPA) sequences.

[4] Depending on the results of your optimization experiments (Step 2), adjust the concentration of $MgCl_2$ up or down to achieve optimal amplification.

[5] ***Tip:*** The nucleotides in vial 4 may also be used to adjust the DIG-dUTP concentration in the labeling/amplification reaction (see Section 2.2.3.1).

4 Mix the reagents and centrifuge briefly to collect the sample at the bottom of the tube. — 1 min

5 Overlay the reaction reagents with 100 µl of mineral oil to prevent evaporation of the reagents during the thermal cycling process. — –

Tip: If your thermal cycler has a top heater, the oil overlay is not necessary.

▶▶▶

Step	Action	Time
6	Place samples in a thermal cycler and start PCR. Use the optimal amplification conditions determined in Step 2 above. ***Tip:*** Optimal cycling conditions depend on the combination of template, primers, and thermal cycler. For best results, you should optimize the PCR for your template and primer pair (as in Step 2). However, the conditions given below are a good starting point for initial experiments. They are designed for amplification of a 3 kb fragment.	1.5 h

	Temperature	Time	Cycle Number
Initial denaturation (before the first cycle)	95°C	2 min	–
Denaturation **Annealing** **Elongation**	95°C 60°C 72°C	30 s 30 s 40 s	Cycles 1 – 10
Denaturation **Annealing** **Elongation**	95°C 60°C 72°C	30 s 30 s 40 s + additional 20 s for each successive cycle[1]	Cycles 11 – 30
Final elongation	72°C	7 min	–

[1] Increased elongation time is only required for long (= 3 kb) fragments. For amplification of shorter fragments, use the 40 s elongation time for all 30 cycles.

7	Do the following: ▶ Use 5 µl of the reaction mixture to check the results of the reaction (Procedure 2.2.2.2 below). ▶ Hold the remainder of the reaction mixture at 4°C until it is used. *PCR-generated probes are very pure and can be used directly in the hybridization reaction. They do not require any clean up.*	–

2.2.2.2. Checking the Synthesis of Labeled Probe

Step	Action	Time
1	Run a portion (5 µl) of each reaction on an agarose mini gel in TAE or TBE buffer, along with a DNA molecular weight marker.	approx. 30 min
2	Stain the gel with 0.5 µg/ml ethidium bromide (EtBr).	30 min
3	Examine the bands on the gel. If the labeling reaction was successful, you should see that: A. The tPA control probe will have an apparent size of 500 – 550 bp. *The actual size of the amplicon is 442 bp. The presence of DIG in DNA makes it run slower in the gel than unlabeled DNA.* B. Both the labeled experimental probe and the unlabeled control probe should be clearly visible on the gel. C. Your unlabeled control probe will be the predicted size. D. Your labeled experimental probe will migrate slower (*i.e.* appear to be larger) than your unlabeled control probe (due to the presence of DIG). E. The EtBr staining of the labeled DNA will be equal to or somewhat less than that of the unlabeled control DNA (due to the presence of DIG).	–
4	If conditions B–E in Step 3 were satisfied, your probe has been labeled successfully (see Figure 10). Use it at the recommended concentration **(2 µl/ml hybridization solution)** in blot hybridization protocols. *If the amount of labeled PCR product band is very strong on the gel, you can use as little as 0.5 µl probe per ml hybridization buffer. If the signal is very faint, use up to 4 µl probe per ml hybridization buffer.*	–

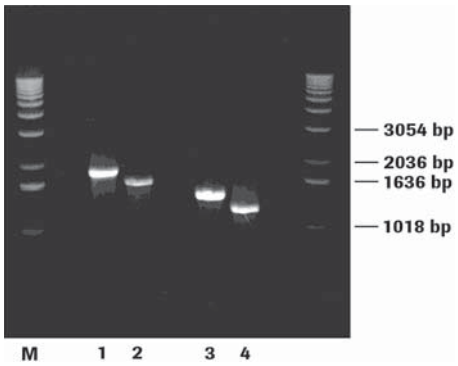


Figure 10. Evaluation of PCR-labeled probes by agarose gel electrophoresis. On this agarose gel, the labeled PCR product has a significantly greater molecular weight than the unlabeled products. (This is due to high density labeling with DIG.)

Result: The labeled and unlabeled products migrate as separate bands on an electrophoretic gel. If you see the shift in molecular weight and the amount of labeled probe is approximately equal (or slightly less than the amount of unlabeled probe, the labeling reaction was successful. You can use the recommended amount of probe (2 µl per ml hybridization buffer) in the hybridization reaction.

2.2.2.3. What To Do Next

IF you want to...	THEN...
See typical results achieved with probes labeled by PCR	See Section 2.2.4 on page 73 of this chapter.
Use your labeled probe to detect DNA on a Southern blot or a dot blot	Go to Section 3.1, "Hybridization of DNA Probes to a Southern Blot," on page 94 of this chapter.
Use your labeled probe to detect recombinant clones in a bacterial or phage library	Go to Section 6.1, "Use of DIG-labeled Probes for Colony and Plaque Hybridization," on page 131 of this chapter.
Store your labeled probe for later use	Store the labeled probe at –20°C. ***Tip:*** Probe will be stable for at least one year at –20°C.

2.2.3 Getting the Best Results from PCR Labeling

2.2.3.1. Critical Hints about PCR Labeling

Factor	Hints
PCR conditions	▶ Optimize PCR amplification parameters (cycling conditions, template concentration, primer sequence, and primer concentration) for each template and primer set in the **absence** of DIG-dUTP before attempting incorporation of DIG.
Template	▶ For best results, use cloned inserts as template. Genomic DNA can be more difficult to use. ▶ Template concentration is crucial to successful production of specific probes (see Figure 11).
Labeling	▶ The PCR DIG Probe Synthesis Kit requires less optimization than most labeling methods, because it contains the Expand High Fidelity Enzyme Blend. This enzyme blend: ▶ Can efficiently use GC-rich regions as template, and ▶ For most templates, requires no optimization of $MgCl_2$ concentration; that is, most labeling reactions will work with the standard concentrations of 1.5 mM $MgCl_2$. ▶ Some DNA templates (especially those with high GC content or longer templates) are not efficiently amplified in the presence of the "standard" concentration of DIG-dUTP (*i.e.*, 0.07 mM, when 5 µl PCR DIG Probe Synthesis Mix of (vial 2) is added to a 50 µl reaction). For these templates, use the nucleotide shock solution (vial 4) of the PCR DIG Probe Synthesis Kit to vary the concentration of DIG-dUTP in the reaction. (see Section 2.2.3.2 below for details on the best nucleotide ratios to use.)

2.2.3.2. Troubleshooting the PCR Labeling Procedure

Problem ☹	Possible Cause 😐	Recommendation ☺
Low yield of DIG-labeled PCR product	PCR is not optimized	▶ Always optimize the PCR parameters (cycling conditions, template concentration, primer sequence, and primer concentration) for each template and primer set in the **absence** of DIG-dUTP before attempting incorporation of DIG.
	Too much DIG-dUTP in reaction[1]	Reduce the concentration of DIG-dUTP in the reaction. This is especially important for long templates. As a general rule: ▶ The 1:3 ratio (DIG-dUTP:dTTP) in the DIG probe synthesis mix (vial 2 of PCR kit) works well for labeling probes up to 1 kb long. *We have encountered some short (<1 kb) probes (especially those with high GC content) that label better with the 1:6 ratio than with the 1:3 ratio. Therefore, initial labeling experiments for each new <1 kb probe should be performed with both ratios of labeled to unlabeled nucleotide.* ▶ Label 1 – 3 kb probes in the presence of a 1:6 ratio of DIG-dUTP: dTTP. *You can easily create a labeling mixture with a 1:6 ratio by mixing equal parts of PCR DIG Probe Synthesis Mix (vial 2) (1:3 ratio) and dNTP shock solution (vial 4) (no DIG-dUTP).* ▶ Label >3 kb probes with even lower concentrations of DIG-dUTP (from a high of 1:6 to a low of 1:10). ***Tip:*** *For labeling of >3 kb probes, use the Expand Long Template System in place of the Expand High Fidelity System supplied in the PCR kit.* *The lower concentrations of DIG listed above will not influence the final sensitivity of the hybridization probe.* ***Tip:*** *Do* **not** *use the 1: 20 ratio of the PCR DIG Labeling Mix for these labeling reactions. It* **will** *affect the sensitivity of the probe.*
Hybridization smear on the blot	Template concentration too high during PCR[2]	▶ For best results, use only small amounts of template. Ideal amounts: 10 pg plasmid DNA or 10 ng genomic DNA. ▶ If possible, use cloned inserts rather than complex genomic DNA as template.
	Other causes	▶ See "Troubleshooting the Hybridization Blot," Section 3.3.2 on page 114 of this chapter.

[1] DIG-labeled nucleotides in the template slow the polymerase and eventually reduce the ability of the polymerase to synthesize full-length products that contain the primer sequences needed for the start of the next round of amplification. As the template gets longer, the effect increases.

[2] Too much template will lead to co-amplification of primary extension products (those copied past the priming sites). These primary extension products may contain repetitive sequences or unrelated products from secondary priming sites (if prepared from genomic DNA) or vector sequences (if prepared from plasmid DNA). In subsequent hybridization assays, the probe-target hybrid will be a smear because the probe will cross-hybridize with vector or genomic DNA sequences. See Figure 11 for an example of the effects of too much template.

3

2.2.3.3. Template Concentration is the Most Critical Factor in Producing Specific Probes

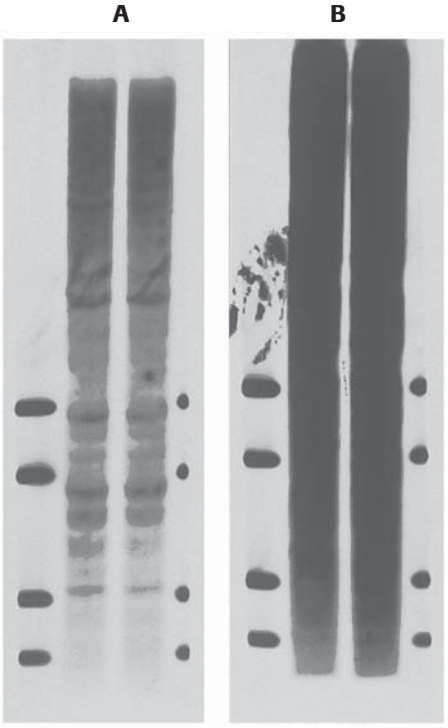


Figure 11. Effect of Template DNA Concentration on the Specificity of Hybridization Probes Generated from Human DNA by PCR. This experiment demonstrates that template concentration during PCR is extremely crucial for creation of high specificity probes. Two probes (for detecting tPA sequences, and cystic fibrosis-related sequences) were prepared directly from 500 ng of human genomic DNA. (This is an amount routinely used in other PCR applications, but is fiftyfold more than the amount recommended for DIG labeling.) The template DNA was predigested with *Not* I to create long restriction fragments that would facilitate the PCR reaction. After labeling, the labeled probe was used to detect sequences in 2.5 µg and 5 of *Pvu* II-digested human genomic DNA on a Southern blot. Standard stringent conditions were used for the hybridization. Hybrids were detected immunochemically and visualized with the chemiluminescent substrate CSPD (2 h exposure).

Result: The hybridization pattern contains many bands, both in the 2.5 µg sample (A) and the 5.0 µg sample (B). In fact, the band pattern resembles a DNA fingerprint. We conclude that the probes have lost their specificity because they contain a high concentration of primary extension products that contain repetitive elements (leading to the "fingerprint" pattern). The probes may also contain significant amounts of product generated from secondary priming sites. The presence of high amounts of both repetitive elements and secondary products is a consequence of the high initial template concentration. Even gel purification of these probes did not improve their specificity.

Conclusion: Template concentration during PCR is the most critical factor in producing specific probes. For most templates, use no more than 10 ng genomic DNA (or 10 pg cloned plasmid DNA) in the PCR.

2.2.4 Typical Results with DIG-labeled Probes Generated by PCR

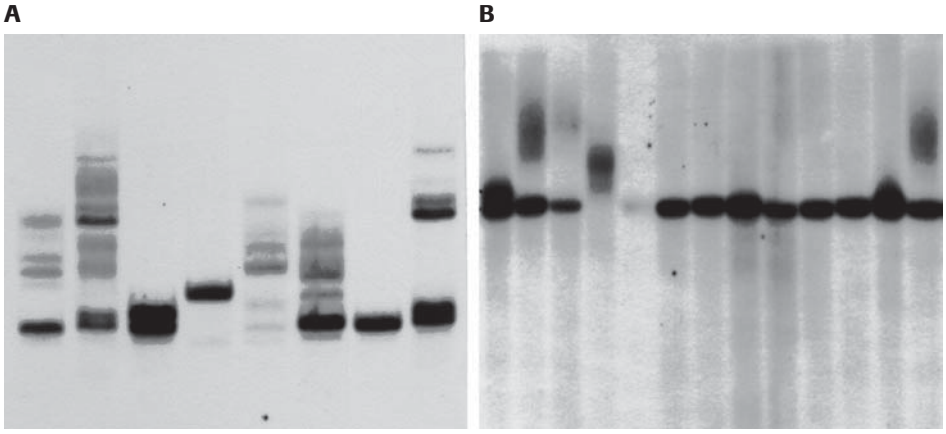


Figure 12. Detection of Fragile X with a DIG-Labeled Probe. Fragile X syndrome is a hereditary disease that leads to an increase in the number of CGG triple repeats on the X chromosome. Since the syndrome affects the X chromosome only, the symptoms of the disorder appear mostly in males, with females being asymptomatic carriers.

When the number of triple repeats becomes too high, the repeat region becomes unstable and prone to breaking. Thus, the X chromosome displays a "fragile" pattern when viewed by chromosome analysis. There is also a premutation phase, in which the number of repeats has increased, but the individual is asymptomatic. A diagnostic test for the presence of Fragile X syndrome can be performed on a Southern blot.

This figure shows such a Southern blot test. The DNA from several subjects was hybridized to a 1 kb labeled probe specific for Fragile X DNA. This figure shows the result obtained with normal subjects, a female subject heterozygous for the Fragile X mutation, a male Fragile X subject in the premutation phase, and a male Fragile X subject with the fully developed mutation.

Two different hybridization probes were used for the test, one radioactively labeled with ^{32}P (B), the other DIG-labeled with the PCR DIG Probe Synthesis Kit (A). Standard procedures were used for Southern blot analysis and detection of probe-target hybrids. Note that the target samples are not exactly the same for the experiments with radioactively labeled and DIG-labeled probes. (Data kindly provided by Dr. Halley, Erasmus University, Rotterdam, Netherlands.)

Result: In DNA taken from subjects with the Fragile X syndrome, additional hybridization bands appear as a smear on the blot above the allelic bands found in normal subjects.

The additional Fragile X bands, when localized with the DIG-labeled probe (A), appear on the X-ray film as discrete bands within the diagnostic smear. Chemiluminescent detection of the probe-target hybrids required only 20 min exposure of the film with the chemiluminescent substrate, CDP-*Star*.

The additional Fragile X bands, when localized with the radioactively labeled probe (B), appear as an undifferentiated smear on X-ray film. Detection of the probe-hybrid signal required overnight exposure of the blot to the X-ray film.

Conclusion: The DIG-labeled probe, prepared by a standard PCR labeling technique, could easily detect the Fragile X bands. The bands visualized with the DIG-labeled probe (top panel) were much sharper than the bands created with the radioactive probe (bottom panel). The result was comparable to that obtained with the radioactively labeled probe, but required much less time to obtain.

3

2.3 Transcriptional Labeling of RNA Probes

For some applications, DIG-labeled RNA is a more effective hybridization probe than DIG-labeled DNA (see Section 2.3.4 on page 83). For example, DIG-labeled RNA probes can detect rare mRNAs in nanogram amounts of total RNA (see Figure 15 in Section 4.1.4, on page 121 of this chapter).

These labeled RNA probes are generated by *in vitro* transcription from a DNA template. In the RNA transcription method, DNA is cloned into the multiple cloning site of a transcription vector between promoters for different RNA polymerases (such as T7, SP6, or T3 RNA polymerase). The template is then linearized by cleavage of the vector at a unique site (near the insert). An RNA polymerase transcribes the insert DNA into an antisense RNA copy in the presence of a mixture of ribonucleotides (including DIG-UTP). During the reaction, the DNA can be transcribed many times (up to a hundredfold) to generate a large amount of full-length DIG-labeled RNA copies (10 – 20 µg RNA from 1 µg DNA in a standard reaction). DIG is incorporated into the RNA at approximately every 25 – 30 nucleotides.

This section describes how to use the components of the DIG Northern Starter Kit to label RNA probes (transcription method). Topics in this section include:

2.3.1 Materials Required for RNA Probe Labeling

Product	Catalog Number	Reagents Included or Description
DIG Northern Starter Kit (all-in-one kit, containing reagents needed in this procedure [vials 1 – 4, 6], plus reagents for estimation of probe yield, hybridization and chemiluminescent detection)	12 039 672 910	▶ Labeling mix, 5× conc. (vial 1a) ▶ Transcription buffer, 5× conc. (vial 1b) ▶ SP6 RNA polymerase, 20 U/µl (vial 2)[1] ▶ T7 RNA polymerase, 20 U/µl (vial 3)[1] ▶ T3 RNA Polymerase, 20 U/µl (vial 4)[1] ▶ Anti-DIG-alkaline phosphatase antibody (vial 5)[1] ▶ DNase I, RNase-free (vial 6)[1] ▶ CDP-*Star* chemiluminescent substrate, ready-to-use (vial 7)[1] ▶ Actin RNA probe, DIG-labeled (vial 8)[1] ▶ DIG Easy Hyb Granules (vial 9)[1] ▶ Blocking solution, 10x conc. (vial 10)[1]

[1] These components of the kit are also available as separate reagents. See the ordering information in Chapter 2, page 44, for catalog numbers.

Additional Materials Required for Protocol

Reagent/Supplies/Equipment	Description
For multiple procedures	
Autoclaved, DMDC- or DEPC-treated, double distilled water	▶ For preparation of all reagents needed in procedures (DMDC = dimethyl-dicarbonate; DEPC = diethyl pyrocarbonate)
Oven, 200°C	▶ For baking glassware to ensure inactivation of RNases
RNase ZAP or similar product	▶ For decontaminating lab benches, labware
Powder-free gloves, sterile forceps	▶ For RNase-free handling of lab supplies, reagents
Ice/water bath	▶ For preparing and storing some samples and reagents
Water bath(s)	Temperatures required: ▶ 68°C, for prewarming prehybridization and hybridization buffers ▶ 42°C, for labeling reaction ▶ 37°C, for DNase I digestion
For preparation of template by cloning	
Transcription plasmid(included in the DIG RNA Labeling Kit, Cat. No. 11 175 025 910)	▶ For cloning of sequence to be transcribed (!) *Plasmid must contain RNA polymerase promoter sequence (SP6, T7, or T3)*
Rapid DNA Dephos & Ligation Kit (Cat. No. 04 898 117 001 40 Reactions Cat. No. 04 898 125 001 160 Reactions)	▶ For inserting target sequence in plasmid
Competent *E. coli* cells	▶ For amplification of plasmid
High Pure Plasmid Purification Kit (Cat. No. 11 754 777 001)	▶ Purification of recombinant plasmid from *E. coli*
Restriction enzymes (see our catalog)	▶ For cloning target sequence into transcription plasmid For linearizing plasmid before transcription; should produce 5' overhang
High Pure PCR Product Purification Kit (Cat. No. 11 732 668 001 or 11 732 676 001), or Phenol, chloroform, ethanol	▶ For deproteinizing and concentrating linearized plasmid

▶▶▶

3

Reagent/Supplies/Equipment	Description
For preparation of template by two-step RT-PCR	
High Pure RNA Isolation Kit (Cat. No. 11 828 665 001)	▶ For preparation of total RNA
Reverse transcriptase	▶ For production of template cDNA from total RNA
PCR Nucleotide Mix (Cat. No. 11 581 295 001)*	▶ Each dNTP, 10 mM
Expand High Fidelity^{PLUS} PCR System (Cat. Nos. 03 300 242 001, 03 300 226 001, 03 300 234 001)*	▶ Polymerase mixture for PCR amplification
PCR primers, containing RNA polymerase promoter sequence (SP6, T7, or T3)	▶ For amplification of target cDNA sequence
For transcriptional labeling	
Template DNA	Highly purified, linearized DNA, containing the target sequence to be labeled and an RNA polymerase (SP6, T7, or T3) promoter. Sequence to be labeled should be at least 200 bp–1000 bp long (ideal length, 1 kb). Template DNA may either be 1 µg of a plasmid containing the cloned target sequence and promoters, or 100 – 200 ng of a specially prepared PCR product. ⚠ *Purity of template is very critical for successful labeling. Follow guidelines given in the procedures below for preparing template.*
Stop buffer	EDTA, 0.2 M, pH 8.0, sterile

* For complete license disclaimer, see page 198

2.3.2 Procedures

The complete RNA labeling protocol involves the procedures listed in the flow chart below. An estimate of the time required for each procedure is also given in the chart.

2.3.2.1 (page 77)	**Purification of Template by Either of Two Methods** Method A: Cloning and plasmid preparation Method B: RT-PCR	3 h – 2 days 2.5 h (+ primer synthesis)
2.3.2.2 (page 80)	**Transcriptional Labeling of Probe**	80 min
2.3.2.3 (page 80)	**Determination of Probe Yield**	2 – 2.5 h

This icon means you can stop the protocol at the end of the procedure and store the product for a time before starting the next procedure.

For all steps of the procedures below, take steps to avoid RNase contamination, including the following:

- Wear powder-free gloves during all steps.
- Use DMDC- or DEPC-treated H_2O for preparing all solutions.
- Autoclave or filter-sterilize all solutions.
- As far as possible, use disposable plasticware, oven-baked (8 h, 200°C) glassware, or plasticware that has been decontaminated with RNase *AWAY* or similar decontaminating solution.
- Rigorously clean lab bench areas with a decontaminating solution.

2.3.2.1. Purification of Template

The template must be a purified DNA containing the sequence to be transcribed into probe and an RNA polymerase promoter (SP6, T7, or T3).

Optimal length of template is approx. 1 kb. Minimum length should be at least 200 bp.

There are two methods of preparing the template:

- Clone the target sequence into a plasmid downstream from an RNA polymerase site, then purify the plasmid and linearize it (standard method).
- Prepare the DNA template from total RNA by RT-PCR and add the required promoter sequence during the PCR step (i.e. include it on the PCR primers).

Each of these procedures is described briefly below.

3

2.3.2.1 A. Standard Method: Prepare DNA template by cloning and plasmid purification

Step	Action	Time
1	Clone the purified target DNA into the multiple cloning site of a transcription vector, downstream from an RNA polymerase (SP6, T7, or T3) promoter site. *For best results, use our restriction enzymes and Rapid DNA Ligation Kit.*	1.0–1.5 h
2	Amplify the plasmid in competent *E. coli* cells.	overnight
3	Purify the plasmid by either of the following methods: ▶ Use density gradient centrifugation in the presence of CsCl, or ▶ Use our High Pure Plasmid Isolation Kit.	16 h, or 30 min
4	Linearize plasmid with a restriction enzyme that leaves a 5' overhang. *If template has 3' overhangs or blunt ends, the transcription reaction may generate wrap-around products that reduce sensitivity and increase background.*	1 h
5	▶ After restriction digest, purify the DNA with either: ▶ Phenol/chloroform extraction, or ▶ Our High Pure PCR Product Purification Kit ▶ Precipitate the purified product with ethanol, then resuspend in sterile, DMDC- or DEPC-treated H_2O or 10 mM Tris at a concentration of 200 – 250 µg DNA/ml	1 h, or 10 min
6	Use 1 µg (4 – 5 µl) of the purified, linearized plasmid as template in Procedure 2.3.2.2 (labeling).	

■

2.3.2.1 B. Prepare DNA template from total RNA by RT-PCR

Step	Action	Time
1	Prepare total RNA by any standard technique, *e.g.* with our High Pure RNA Isolation Kit.	30 min
2	Reverse transcribe the total RNA with oligo(dT) primer and an appropriate reverse transcriptase.	1.25 h
3	Synthesize a pair of PCR primers that contain sequences complementary to the target sequence (×-mer) to be transcribed and the sequence of one of the following RNA promoters:	0.5 – 1 day

RNA Polymerase	Promoter Sequence
SP6	The use of SP6 promotor consensus sequences cannot be recommended for PCR generated DNA fragments because SP6 Polymerase can only initiate efficient transcription if the promotor sequence lies within a plasmid environment.
T7	5'TAATACGACTCACTATAGGG, or 5'TAATACGACTCACTATAGGA
T3	5'AATTAACCCTCACTAAAGGG

▶▶▶

Step	Action	Time

4 To a sterile, RNase-free microcentrifuge tube (on ice), add these reagents (in the order listed): — 5 min

Reagent	Volume	Final Concentration
Double dest. water, RNase-free	variable	
Expand High Fidelity buffer, 10× conc.	5 µl	1×, with 1.5 mM MgCl2
Nucleotide Mix (each dNTP, 10 mM)	1 µl	each dNTP, 0.2 mM
Primer 1 (sense)	variable	300 nM
Primer 2 (antisense)	variable	300 nM
Expand High Fidelity enzyme mix[1]	0.75 µl	2.6 U
cDNA (from Step 2 above)	2 µl	
Total volume	**50 µl**	

[1] Expand High Fidelity enzyme mix can amplify up to 5 kb targets.

5 Gently vortex the contents of the tube to produce a homogeneous solution, then centrifuge briefly to collect the sample at the bottom of the tube. — 1 min

6 Place samples in a thermal cycler and start PCR. Set the temperature profile of the cycler as follows: — 50 min

	Temperature	Time	Number of Cycles
Initial denaturation (before the first cycle)	94°C	2 min	–
Denaturation **Annealing** **Elongation**	94°C 60°C 72°C	45 s 45 s 90 s	30×

7 Use 4 µl of the PCR product (approx. 100 – 200 ng) directly in Procedure 2.3.2.2 (labeling). — –

3

2.3.2.2. Transcriptional Labeling of RNA Probes

<table>
<tr><th>Step</th><th>Action</th><th>Time</th></tr>
<tr><td>1</td><td>To a sterile, RNase-free microcentrifuge tube (on ice), add these reagents (in the order listed):
<table>
<tr><th>Reagent[1]</th><th>Volume</th><th>Final Concentration</th></tr>
<tr><td>Sterile, RNase-free double-distilled H_2O</td><td>variable</td><td></td></tr>
<tr><td>Template DNA (from Procedure 2.3.2.1)</td><td>variable</td><td>Per 20 µl reaction:
▶ 1 µg linearized plasmid DNA
OR
▶ 100 – 200 ng (4 µl) PCR product</td></tr>
<tr><td>Labeling Mix, 5× conc. (vial 1a)</td><td>4 µl</td><td>1×</td></tr>
<tr><td>Transcription buffer, 5× conc. (vial 1b)</td><td>4 µl</td><td>1×</td></tr>
<tr><td>RNA polymerase, 20 U/µl</td><td>2 µl</td><td>2 U/µl of:
▶ SP6 RNA polymerase (vial 2), or
▶ T7 RNA polymerase (vial 3), or
▶ T3 RNA polymerase (vial 4)</td></tr>
<tr><td>Total volume</td><td>20 µl</td><td></td></tr>
</table>
[1] Numbered vials are components of the DIG Northern Starter Kit.</td><td>5 min</td></tr>
<tr><td>2</td><td>Gently vortex contents of the tube to produce a homogeneous solution, then centrifuge briefly to collect sample at bottom of tube.</td><td>1 min</td></tr>
<tr><td>3</td><td>Incubate the tube at 42°C for 1 h.</td><td>1 h</td></tr>
<tr><td>4</td><td>(optional)
▶ Add 2 µl DNase I (RNase-free, vial 6) to the tube.
▶ Incubate at 37°C for 15 min to remove template DNA.</td><td>15 min</td></tr>
<tr><td>5</td><td>Add 2 µl 0.2M EDTA (pH 8.0) to the tube and mix thoroughly to stop the reaction.</td><td></td></tr>
<tr><td>6</td><td>▶ Use 0.5 µl of the reaction mixture to estimate the amount of probe you have made (see topic 2.3.2.3 below).
▶ Hold the remainder of the reaction mixture at –20°C until it is used.
RNA transcripts can be analyzed by agarose gel electrophoresis and ethidium bromide staining (but this cannot replace 2.3.2.3.)</td><td></td></tr>
</table>

2.3.2.3. Determination of Probe Yield

No hybridization can be successful unless an optimal amount of probe is used. Quantification of the labeled probe is the first step in determining the optimal amount of probe. Therefore, after completion of the labeling reaction, the next step is determining how much labeled probe you made. This determination **must** be done before the probe can be used successfully in a hybridization reaction.

To learn how to do estimate the yield of your labeled probe, go to Section 2.5, "Estimation of Probe Yield by the Direct Detection Method" on page 85 of this chapter.

2.3.2.4. What To Do Next

IF you want to...	THEN...
See typical results achieved with RNA probes	See Section 2.3.4 on page 83 of this chapter.
Use your labeled probe to detect RNA on a northern blot	Go to Section 3.2, "Hybridization of RNA Probes to a Northern Blot," on page 103 of this chapter.
Use your labeled probe to detect recombinant clones in a bacterial or phage library	Go to Section 6.1, "Use of DIG-labeled Probes for Colony and Plaque Hybridization," on page 131 of this chapter.
Store your labeled probe for later use	Store the labeled probe at –20° or –70°C in ethanol. ***Tip:*** Labeled probe will be stable at –20° or –70°C for at least one year.

2.3.3 Getting the Best Results from Transcriptional Labeling

2.3.3.1. Critical Hints about RNA Probe Labeling

Factor	Hints
RNases	RNases are ubiquitous and do not require any cofactors for activity. If you want to be successful, take all possible precautions to prevent RNase contamination. For instance: ▶ As far as possible, use disposable plasticware, oven-baked glassware, or plasticware that has been decontaminated with RNase ZAP or similar reagents. ▶ Prepare all solutions with water that has been treated with diethyl pyrocarbonate (DEPC) or dimethyl-dicarbonate (DMDC). If possible, autoclave the solutions. ▶ Wear gloves throughout the procedure.
Template purity	▶ Labeling efficiency depends greatly on the purity of the DNA template. Template should be highly purified. ▶ The final template must be linearized, phenol/chloroform extracted and ethanol precipitated.
Template sequence	▶ Some primer and/or polylinker regions in DNA templates are homologous to portions of the ribosomal 28s and 18s RNA sequences. Therefore, labeled probes may generate specific, but unwanted signals in samples that contain these prominent RNAs. To minimize this effect, remove as much of the polylinker sequences from your template as possible. ▶ If you use PCR to make the DNA template (Procedure 2.3.2.1B), the product of the Expand High Fidelity reaction contains some fragments with a single 3' A overhang. This overhang may produce wrap-around products in the transcriptional labeling reaction.
Template length	▶ Optimal template length is approx. 1 kb. ▶ Minimum length should be at least 200 bp.
Storage of probe	▶ For long term stability, RNA probes should be aliquoted and stored at –20°C or –70°C. ▶ DIG-labeled RNA probes are stable for at least 1 year at –20°C or –70°C in ethanol.
Probe sensitivity	▶ To quickly determine the sensitivity of a DIG-labeled antisense RNA probe, prepare the corresponding sense RNA (unlabeled) by *in vitro* transcription. Then use the purified sense transcript at varying concentrations as target on a Northern blot (see Section 3.2 in this chapter). From the result of the blot (see Figure 14 on page 83), you can easily determine the lowest amount of target (sense transcript) that can be detected by labeled probe (antisense transcript).

2.3.3.2. Troubleshooting the RNA Labeling Procedure

Problem ☹	Possible cause 😐	Recommendation ☺
Inefficient probe labeling	Insufficient template	Start with: ▶ 1 µg linearized plasmid DNA, or ▶ 100 – 200 ng PCR product
	Template degraded	▶ Follow strict procedures to prevent nuclease contamination during isolation of template.
	EDTA in template preparation	▶ EDTA inhibits the labeling reaction. Do not store template in buffers containing more than 0.1 mM EDTA.
	Protein in template preparation	▶ Remove proteins (especially RNases) from template DNA by phenol/chloroform extraction and ethanol precipitation.
	Plasmid template not linearized	▶ Linearize plasmid template with a restriction enzyme before labeling.
High background during chemiluminescent detection (Section 4.1 in this chapter)	Inefficient probe labeling	▶ See above comments under "Inefficient probe labeling"
	Other causes	▶ See "Troubleshooting the Hybridization Blot," Section 3.3.2 on page 114 of this chapter.

2.3.4 Typical Results with DIG-labeled RNA probes

For more results with RNA probes, see Section 4.1.4, page 121, in this chapter.

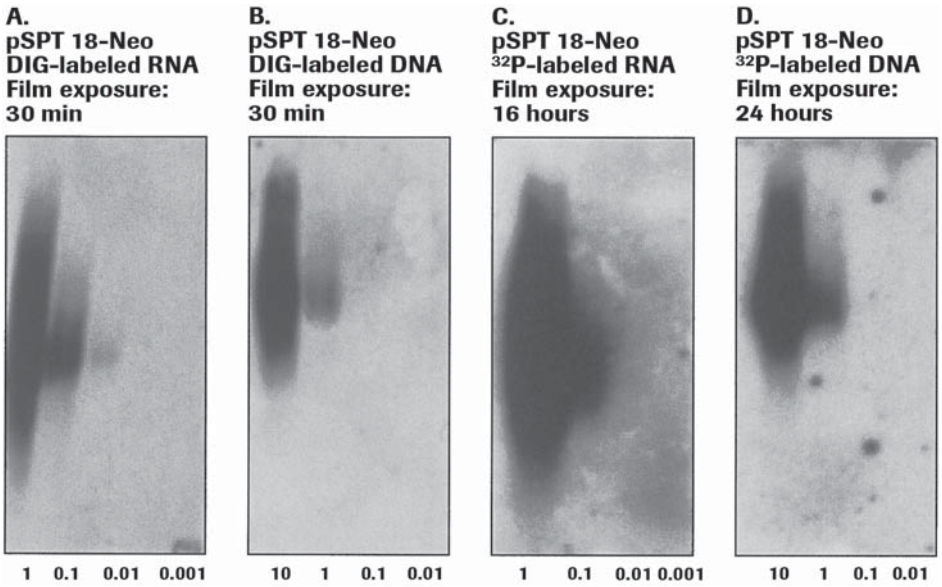


Figure 13. Comparison of Sensitivity of DNA and RNA Probes on Northern Blots. A model Northern blot system was designed to show the relative sensitivity of DIG-labeled DNA (B), DIG-labeled RNA (A), radioactively labeled RNA (C), and radioactively labeled DNA (D) as probes for detecting RNA targets. The target samples contained various amounts of poly(A)$^+$ *neo* RNA (sense strand), which was transcribed *in vitro*. The number of pg RNA in each sample is indicated at the bottom of each lane.

The hybridization probes contained either the antisense strand of neo RNA or the corresponding cDNA. The hybridization procedures were optimized for the type of probe used. Radioactive signals were visualized by exposure of the blot to X-ray film for 16 h (RNA) or 24 h (DNA). DIG signals were visualized by chemiluminescent substrate addition and exposure of the blot to X-ray film for 30 min (RNA or DNA).

Result and conclusion: The DIG-labeled RNA probe could detect as little as 0.01 ng of target RNA after a 30 min exposure. In contrast, the radioactively labeled RNA probe could only detect 0.1 ng after a 16 h exposure. Both the DIG-labeled DNA probe and the radioactively labeled DNA probe could detect 1 ng of target RNA. Thus, DIG-labeled RNA probes are a hundredfold more sensitive than DIG-labeled DNA probes for detecting RNA targets. In addition, the DIG-labeled probe (DNA or RNA) is more sensitive than the corresponding radioactively labeled probe. (Note that, although the two DNA probes detected the same amount of target, the DIG signal was visible in just 30 min, while the radioactive signal required 24 h.)

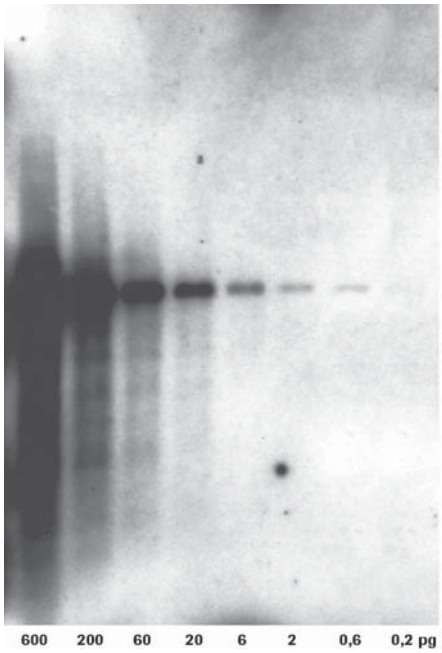


Figure 14. Evaluating Sensitivity of DIG-labeled RNA Probes on a Northern Blot. Varying amounts of unlabeled sense RNA transcript (600 – 0.2 pg) from a purified single copy gene were separated on a MOPS-formaldehyde gel and transferred to a nylon membrane by Northern blotting. The sensitivity of the DIG-labeled hybridization probe (corresponding antisense RNA transcript from the same gene) was determined in a standard hybridization reaction (as described in Section 3.2 of this chapter). The conditions were: overnight hybridization at 65°C with 100 ng probe per ml DIG Easy Hyb, followed by stringent washes. The hybrids were visualized with CSPD chemiluminescent substrate. The chemiluminescent assay was exposed to X-ray film for 35 min. (Data courtesy of M. Block, University of Hamburg, Germany.)

Conclusion: The labeled probe can detect as little as 0.2 pg target RNA (last lane on right).

3

2.4 DIG Oligonucleotide Labeling

For some applications, such as *in situ* hybridization, a DIG-labeled synthetic oligonucleotide is the best hybridization probe. In addition to *in situ* hybridizations, DIG-labeled oligonucleotides may be used:

- As hybridization probes in
  - Dot/slot blots
  - Library screening
  - Detection of repeated gene sequences on Southern blots

    Oligonucleotide probes are sensitive enough to detect single copy gene sequences in complex genomes if sufficient target DNA (e.g. 10 µg human genomic DNA) is present on the blot.
  - Detection of abundant mRNAs on Northern blots

Several methods are available for DIG-labeling oligonucleotides. These are summarized in the table below.

Different Methods for Labeling Oligonucleotides with DIG

Method[1,2] (Key Roche Applied Science Product)	Amount of Oligonucleotide[3] Needed	Labeling Time and Temperature	Labeled Probe Can Detect	Advantages of Method
Labeling 3' end with DIG-ddUTP (DIG Oligonucleotide 3' End Labeling Kit, 2nd generation Cat. No. 03 353 575 910)	100 pmol	15 min, 37°C	10 pg DNA	▶ Requires only a small amount of template ▶ Labeled probes can be used without purification ▶ Reaction can be scaled up indefinitely (if you increase incubation time to 1 h)
Adding a 3' tail of DIG-dUTP and dATP (approx. 40–50 residues) (DIG Oligonucleotide Tailing Kit, 2nd generation Cat. No. 03 353 583 910)	100 pmol	15 min, 37°C	1 pg DNA	▶ Requires only a small amount of template ▶ Produces more sensitive probes than end labeling ▶ Labeled probes can be used without purification ▶ Reaction can be scaled up indefinitely

[1] All of the oligonucleotide labeling methods generate probes that are suitable for library screening, dot/slot blots, and in situ assays. They may also be used to detect DNA sequences on Southern blots or abundant mRNA sequences on Northern blots.

[2] All labeling reactions are simple procedures. For details of these labeling reactions, see the package inserts for the appropriate Roche product.

[3] Purity of the oligonucleotide is important. Always purify the oligonucleotide by HPLC before labeling it.

[4] Efficient labeling of oligonucleotides requires 60 min at 85°C, longer than the standard (30 min) incubation listed in the package insert for DNA or RNA.

2.5 Estimation of Probe Yield by the Direct Detection Procedure

To add the correct amount of probe to a hybridization, you must first determine the amount of DIG-labeled probe produced in the labeling reaction. The direct detection procedure given here compares the amount of DIG label in a series of dilutions prepared from the labeled probe with a known concentration of a DIG-labeled control nucleic acid.

If you label a DNA probe by PCR, you do not need to perform a direct detection to evaluate the yield. For PCR-labeled probes, use the gel electrophoresis evaluation method (Section 2.2.2.2, page 69 of this chapter).

2.5.1 Materials Required for Direct Detection Procedure

Reagent/Supplies/Equipment	Reagents Included or Description
DIG Wash and Block Buffer Set (DNase- and RNase-free) (Cat. No. 11 585 762 001)	▶ Washing buffer, 10× conc. ▶ Maleic Acid buffer for dilution of blocking solution, 10× conc. ▶ Blocking solution, 10× conc. ▶ Detection buffer, 10× conc.
Nucleic acid dilution buffers	▶ **DNA Dilution Buffer:** 50 µg/ml herring sperm DNA in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0 at 25°C)[1,2] ▶ **RNA Dilution Buffer:** Mixture (5:3:2) of DMDC- or DEPC-treated double-distilled H_2O: 20× SSC: formaldehyde
Nucleic acid controls	▶ DIG-labeled Control DNA, 5 ng/µl (Cat. No. 11 585 738 910)[1] ▶ DIG-labeled actin control RNA, 10 ng/µl (Cat. No. 11 498 045 910)[3] ▶ DIG-labeled control oligonucleotide, 3' end-labeled, 2.5 pmol/µl (Cat. No. 11 585 754 910)[2] ▶ DIG-tailed control oligonucleotide, 2.5 pmol/µl (component of DIG Oligonucleotide Tailing Kit)
Anti-Digoxigenin, alkaline phosphatase conjugated	▶ Fab fragments (Cat. No. 11 093 274 910)[1,3]
Chemiluminescent alkaline phosphatase substrate	Choose either: ▶ Ready-to-use CSPD (Cat. No. 11 755 633 001)[1] ▶ Ready-to-use CDP-*Star* (Cat. No. 12 041 677 001)[3]
Nylon Membranes, Positively Charged	▶ Cat. No. 11 417 240 001 (0.3 × 0.3 m roll) ▶ Cat. No. 11 209 272 001 (20 × 30 cm) ▶ Cat. No. 11 209 299 001 (10 × 15 cm)
UV transilluminator or UV crosslinker	▶ To fix nucleic acid to nylon membrane

[1] This reagent is also included in the DIG-High Prime DNA Labeling and Detection Starter Kit II.

[2] This reagent is also included in the DIG Oligonucleotide 3' End Labeling Kit.

[3] This reagent is also included in the DIG Northern Starter Kit.

2.5.2 Procedures

The procedures required for direct detection involves the procedures listed in the flow chart below. An estimate of the time required for each procedure is also given in the chart.

2.5.2.1 (page 87)	**Preparing serial dilutions of labeled probe and spotting them on a nylon membrane**	15 min

2.5.2.2 (page 91)	**Detecting DIG in spots withchemilumi-nescence**	2 – 2.5 h

This icon means you can stop the protocol at the end of the procedure and store the product for a time before starting the next procedure.

Before you start: Make the working solutions required for this assay:

Working Solution	Composition/Preparation	Storage/Stability
Maleic Acid Buffer[1]	0.1 M Maleic acid, 0.15 M NaCl; adjust with NaOH (solid) to pH 7.5 (20°C)	room temperature, stable
Blocking Solution[1]	Dilute 10x Blocking Solution 1:10 with Maleic Acid Buffer	Prepare fresh (stable at 4°C)
Washing Buffer[1]	0.1 M Maleic acid, 0.15 M NaCl; pH 7.5; 0.3% (v/v) Tween 20 *Shake the stock solution to suspend the buffer components before diluting.*	room temperature, stable
Antibody Solution[2]**, 75 milliunits/ml**	Centrifuge Anti-DIG-alkaline phosphatase (vial 5) for 5 min at 10 000 rpm in the original vial. Remove an aliquot from the supernatant and dilute 1:10^4 with Blocking Solution.	Prepare fresh (stable 12 h, 4°C)
Detection Buffer[1]	0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20°C)	room temperature, stable

[1] RNase-free, 10× concentrated stock solutions of these reagents are available in the DIG Wash and Block Buffer Set (11 585 762 001). Unless otherwise indicated, dilute them tenfold with double distilled water (DMDC- or DEPC-treated, if working with RNA probes) to prepare the working solutions.

[2] Stock (undiluted) antibody also available in DIG-High Prime Starter Kit II and DIG Northern Starter Kit.

2.5.2.1. Preparing Serial Dilutions of Labeled Probe and Spotting them on a Membrane

Step	Action	Time
1	Estimate yield of labeled probe, which depends upon the labeling time and the initial concentration of template. (See appropriate labeling procedure for information on expected yield.)	

***Examples*:**

IF your template was...	AND you labeled by this method...	THEN, your expected yield of probe is...
300 ng highly purified DNA	random primed labeling with DIG High Prime (1 h incubation)	450 ng labeled DNA (final concentration = 450 ng/22 µl = 20 ng/µl)
1 µg highly purified plasmid	transcriptional labeling (1 h incubation)	20 µg labeled RNA (final concentration = 20 µg/24 µl = 800 ng/µl)
100 pmol oligonucleotide 3' ends	3' end labeling (15 min incubation)	100 pmol end-labeled oligonucleotide (final concentration = 100 pmol/22 µl = 4.5 pmol/µl)

2 Do one of the following:

IF you have...	THEN go to...
DNA probe	Step 3A
RNA probe	Step 3B
Oligonucleotide probe	Step 3C

▶▶▶

Step	Action	Time
3 A	Based on the expected yield of probe and the final volume of the reaction mixture, prepare serial dilutions of your DNA probe as follows: ▶ Prepare a 1 ng/µl working solution of the labeled DNA probe with DNA Dilution Buffer. ***Example:*** *Make a 1:20 dilution (1 µl labeled product + 19 µl DNA Dilution Buffer) of the DNA probe in the Step 1 example (original concentration = 20 ng/µl).* ▶ Prepare a 1 ng/µl working solution of DIG-labeled Control DNA (original concentration = 5 ng/µl) in DNA Dilution Buffer. ▶ Using the DNA Dilution Buffer, prepare separate serial dilutions of the labeled probe and the control probe, as shown in the table below:	5 min

Tube	DNA (µl)	From Tube #	DNA Dilution Buffer (µl)	Overall Dilution (from Tube D1)	Final Concen-tration
D1*		-		None	1 ng/µl
D2	2	D1	198	1:100	10 pg/µl
D3	15	D2	35	1:330	3 pg/µl
D4	5	D2	45	1:1000	1 pg/µl
D5	5	D3	45	1:3300	0.3 pg/µl
D6	5	D4	45	$1:10^4$	0.1 pg/µl
D7	5	D5	45	1:33000	0.03 pg/µl
D8	5	D6	45	$1:10^5$	0.01 pg/µl
D9	0	-	50	-	0

* Working solution of labeled probe or control DNA.

▶▶▶

Step	Action	Time

3 B

Based on the expected yield of probe and the final volume of the reaction mixture, prepare serial dilutions of your RNA probe as follows:

- Prepare a 10 ng/µl working solution of the labeled RNA probe with RNA Dilution Buffer.
  Example: *Make a 1:80 dilution (1 µl labeled product + 79 µl RNA Dilution Buffer) of the RNA probe in the Step 1 (original concentration = 800 ng/µl).*
- As control, use the DIG-labeled actin Control RNA (10 ng/µl).
- Using the RNA Dilution Buffer, prepare separate serial dilutions of the labeled probe and the control probe, as shown in the table below:

Tube	RNA (µl)	From Tube #	RNA Dilution Buffer (µl)	Overall Dilution (from Tube R1)	Final Concentration
R1*		-		None	10 ng/µl
R2	2	R1	18	1:10	1 ng/µl
R3	2	R2	198	1:1000	10 pg/µl
R4	15	R3	35	1:3300	3 pg/µl
R5	5	R3	45	$1:10^4$	1 pg/µl
R6	5	R4	45	$1:3.3 \times 10^4$	0.3 pg/µl
R7	5	R5	45	$1:10^5$	0.1 pg/µl
R8	5	R6	45	$1:3.3 \times 10^5$	0.03 pg/µl
R9	5	R7	45	$1:10^6$	0.01 pg/µl
R10	0	-	50	-	0

* Working solution of labeled probe or control RNA.

5 min

3 C

Based on the expected yield of probe and the final volume of the reaction mixture, prepare serial dilutions of your oligonucleotide probe as follows:

- Prepare a 100 fmol/µl working solution of the labeled oligonucleotide
  Example: *Make a 1:45 dilution (1 µl labeled product + 44 µl DNA Dilution Buffer) of the oligonucleotide probe in the Step 1 example (original concentration = 4.5 pmol/µl).*
- As a control, make a 1:25 dilution of the DIG-labeled control oligonucleotide solution (original concentration = 2.5 pmol/µl).
- Using the DNA Dilution Buffer, prepare separate serial dilutions of the labeled probe and the control probe, as shown in the table below:

Tube	Oligo (µl)	From Tube #	DNA Dilution Buffer (µl)	Overall Dilution (from Tube N1)	Final Concentration
N1*			-	None	100 fmol/µl
N2	3	N1	7	1:3.3	30 fmol/µl
N3	2	N1	18	1:10	10 fmol/µl
N4	2	N2	18	1:33	3 fmol/µl
N5	2	N3	18	1:100	1 fmol/µl
N6	0	-	20	-	0

* Working solution of labeled probe or control oligonucleotide.

5 min

▶▶▶

Step	Action	Time
4	On a narrow strip (approx. 3 × 5 cm) of Positively Charged Nylon Membrane: ▶ Apply 1 µl spots from probe dilutions D2 – D9 (from Step 3A), R3 – R10 (from Step 3B), or N2 – N6 (from Step 3C). ▶ In a row parallel to the probe dilutions, apply 1 µl spots of the corresponding control dilutions (*i.e.*, D2 – D9, R3 – R10, or N2 – N6, made from the appropriate control). ▶ Mark location of each probe and control spot with a pencil.	5 min
5	Fix the nucleic acid spots to the membrane by doing one of the following: ▶ Crosslink with Stratalinker 120 mJ, or ▶ Crosslink with UV light for 3–5 min, or ▶ Bake the membrane at 120°C for 30 min, or ▶ Bake the membrane at 80°C for 2 h	3 – 5 min, or 30 min, or 2 h

2.5.2.2. Detecting DIG in Spots with Chemiluminescence

Before beginning this procedure, have all required reagents made and ready to use. This will prevent drying of the membrane during the procedure and minimize background problems. Unless otherwise indicated, all steps are performed at room temperature (15° – 25°C).

Step	Action	Time
1	▶ Transfer the membrane (from Procedure 2.5.2.1) to a plastic container (*e.g.*, a petri dish) containing 20 ml Washing Buffer. ▶ Incubate for 2 min with shaking. ▶ Discard the Washing Buffer.	2 min
2	▶ Incubate membrane for 30 min in 10 ml Blocking Solution. ▶ Discard the Blocking Solution.	30 min
3	Incubate the membrane for 30 min in 10 ml Antibody Solution.	30 min
4	Wash membrane twice (2 × 15 min) with 10 ml portions of Washing Buffer.	30 min
5	Equilibrate membrane 2 – 5 min in 10 ml Detection Buffer.	2 – 5 min
6	▶ Place the membrane inside a hybridization bag (or other tightly sealable envelope-like container) with the DNA side facing up. *Do not wrap membrane in plastic wrap, which cannot be tightly sealed.* ▶ Apply 0.1 ml (about 4 drops) of ready-to-use CSPD or CDP-*Star* across the surface of the membrane. ▶ **As you are applying the substrate, immediately** cover the dampened part of the membrane with the second side of the bag so the substrate is spread evenly over the membrane. Do not let air bubbles form between the membrane and the upper surface of the bag.	1 min
7	▶ Incubate membrane for 5 min. ▶ Squeeze excess liquid out of the bag and seal the sides of the bag close to the membrane.	5 min
8	Do one of the following: ▶ If you are using CSPD, incubate membrane for 10 min at 37°C. ▶ If you are using CDP-*Star*, skip this step and go to Step 9.	5 – 10 min
9	Expose the sealed envelope (containing the membrane) ▶ Lumi-Film X-ray film (15 – 25 min)	5 – 25 min
10	▶ Look at the spots containing control dilutions. Depending on the type of nucleic acid you are analyzing, can you see: ▶ The fifth DNA control spot (Tube D6, 0.1 pg/µl), or ▶ The fifth RNA control spot (Tube R7, 0.1 pg/µl), or ▶ The fourth oligonucleotide control spot (Tube N5, 1 fmol/µl) ▶ Based on your answer, do one of the following:	

IF you answered...	THEN...
YES	Go to Step 11.
NO	Repeat Step 9, but expose for a longer time, until you see the D6, R7, or N5 control spot.

▶▶▶

Step	Action	Time
11	Analyze the results by looking at the intensity of the spots containing probe dilutions, as follows:	1 min

IF the most diluted spot you can see is...	THEN your labeling reaction produced...
(for DNA samples)	
The fifth DNA probe spot (Tube D6)	The **expected** amount of labeled DNA. ***Example:*** *The DNA sample diluted in Step 3A, Procedure 2.5.2.1, would actually contain 20 ng labeled probe per µl.*
The fourth DNA probe spot (Tube D5)	An **adequate** amount of labeled DNA. ***Example:*** *The DNA sample diluted in Step 3A, Procedure 2.5.2.1, would actually contain approx. 7 ng labeled probe per µl.*
The third DNA probe spot (Tube D4)	An **inadequate** amount of labeled DNA. *After deciding DNA probe yield, go to Step 12.*
(for RNA samples)	
The fifth RNA probe spot (Tube R7)	The **expected** amount of labeled RNA. ***Example:*** *The RNA sample diluted in Step 3B, Procedure 2.5.2.1, would actually contain 800 ng labeled probe per µl.*
The fourth RNA probe spot (Tube R6)	An **adequate** amount of labeled RNA probe. ***Example:*** *The RNA sample diluted in Step 3B, Procedure 2.5.2.1, would actually contain 270 ng labeled probe per µl.*
The third RNA probe spot (Tube R5)	An **inadequate** amount of labeled RNA. *After deciding RNA probe yield, go to Step 12.*
(for oligonucleotide samples)	
The fourth oligo probe spot (Tube N5)	The **expected** amount of labeled oligo. ***Example****: The oligo sample diluted in Step 3C, Procedure 2.5.2.1, would actually contain 4.5 pmol labeled probe per µl.*
The third oligo probe spot (Tube N4)	An **adequate** amount of labeled oligo. ***Example****: The oligo sample diluted in Step 3C, Procedure 2.5.2.1, would actually contain approx. 1.5 pmol labeled probe per µl.*
The second oligo probe spot (Tube N3)	An **inadequate** amount of labeled oligo. *After deciding oligonucleotide probe yield, go to Step 12.*

▶▶▶

Step	Action	Time
12	On the basis of the results in Step 11, do one of the following:	–

IF your reaction made...	THEN...
The **expected** amount of labeled probe (*i.e.*, Tube D6, R7, or N5 spot visible in Step 11)	Use the calculated concentration of probe to determine how much probe solution to add to a hybridization. ***Example*:** *For the DNA sample used in Step 3A, Procedure 2.5.2.1, use 1.25 µl of the 20 ng/µl DNA probe solution (25 ng DNA probe) per ml hybridization buffer to detect DNA on a Southern blot.*
An **adequate** amount of labeled probe (*i.e.*, Tube D5, R6, or N4 spot visible in Step 11)	Use the calculated concentration of probe to determine how much probe solution to add to a hybridization. ***Example*:** *For the DNA sample used in Step 3A, Procedure 2.5.2.1, use 3.75 µl of the approx. 7 ng/µl DNA probe solution (25 ng DNA probe) per ml hybridization buffer to detect DNA on a Southern blot.*
An **inadequate** amount of labeled probe(*i.e.*, Tube D5, R6, or N4 spot not visible in Step 11)	Discard the labeled probe and make a new one.

3. Techniques for Hybridization of DIG-labeled Probes to a Blot

Use the techniques in this section to localize target nucleic acids on a blot with one of the probes you labeled in Section 2. The methods described in this section include:

For information on this topic	Turn to Section	Starting om page
Hybridization of DNA Probes to a Southern Blot	3.1	94
Hybridization of RNA Probes to a Northern Blot	3.2	103
Getting the Best Results from Blots	3.3	112

3.1 Hybridization of DNA Probes to a Southern Blot

A DIG-labeled probe is great for detecting DNA samples on Southern blots and dot blots. This section describes how to separate genomic DNA electrophoretically, transfer the DNA to a nylon membrane (Southern blot), and use DIG-labeled probes to detect a single copy gene on the blot. The hybridization probe for this procedure can be prepared by either random primed labeling (Section 2.1, page 57 of this chapter) or PCR labeling (Section 2.2, page 64 of this chapter).

Oligonucleotide probes can also be used to detect DNA abundant targets. For more information, see "How To Use DIG-labeled Oligonucleotide Probes To Detect DNA Targets on a Southern Blot" in Section 3.1.3 (page 102).

Topics in this section include:

For information on this topic	Turn to page
Materials Required for Electrophoresis, Blotting, and Hybridization	95
Procedures:	
▶ Separating DNA Samples on an Agarose Gel	96
▶ Transferring DNA to Membrane (Southern Blot, Capillary Transfer Method)	97
▶ Prehybridizing the Blot with DIG Easy Hyb	99
▶ Hybridizing the DIG-labeled DNA Probe to DNA on the Blot	100
▶ What To Do Next	102
How To Use DIG-labeled Oligonucleotide Probes To Detect DNA Targets on a Southern Blot	102

3.1.1 Materials Required for Electrophoresis, Blotting, and Hybridization

You will need the following reagents and equipment for the procedures below:

Reagent/Equipment	Description/Product
For Electrophoresis	
Agarose, nucleic acid grade	▶ See Chapter 2, page 48 for ordering information on suitable agaroses
Running buffer	▶ 89 mM Tris borate, 2 mM EDTA, pH 8.3 (TBE) [or other suitable buffer]
DIG-labeled DNA Molecular Weight Marker	▶ For easy determination of target DNA molecular weight on the blot. For available DIG-labeled markers, see the ordering information on page 44.
For the Southern Blot	
Labeled DNA probe	▶ Prepared by either random primed labeling (Section 2.1, page 57 of this chapter) or PCR labeling (Section 2.2, page 64 of this chapter)
Nylon Membranes, Positively Charged	▶ Cat. No. 11 417 240 001 (0.3 × 0.3 roll) ▶ Cat. No. 11 209 272 001 (20 × 30 cm) ▶ Cat. No. 11 209 299 001 (10 × 15 cm)
UV transilluminator or UV crosslinker or oven	▶ To fix DNA to nylon membrane
Water bath	▶ For prewarming prehybridization and hybridization buffers
Shaking water bath	▶ For prehybridization/hybridization incubations, stringent washes
Sterile, double distilled water	▶ For rinsing gel, dilution of DNA
Depurination solution	▶ 250 mM HCl
Denaturation solution	▶ 0.5 M NaOH, 1.5 M NaCl
Neutralization solution	▶ 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl
20× SSC buffer	▶ 3 M NaCl, 300 mM sodium citrate, pH 7.0 (Cat. No. 11 666 681 001)
10× SSC	▶ 1.5 M NaCl, 150 mM sodium citrate, pH 7.0
2× SSC	▶ 0.3 M NaCl, 30 mM sodium citrate, pH 7.0
Whatman 3MM paper	▶ For capillary transfer
For prehybridization and hybridization	
Hybridization buffer	▶ DIG Easy Hyb (Cat. No. 11 603 558 001), or ▶ DIG Easy Hyb granules (Cat. No. 11 796 895 001)[1]
Hybridization bags	▶ Cat. No. 11 666 649 001
Low stringency buffer	▶ 2× SSC containing 0.1% SDS
High stringency buffer	▶ 0.5× SSC containing 0.1% SDS

[1] This reagent is also included in the DIG-High Prime DNA Labeling and Detection Starter Kit II (Cat. No.11 585 614 910).

3

3.1.2 Procedures

The Southern blot protocol involves the procedures listed in the flow chart below. An estimate of the time required for each procedure is also given in the chart.

3.1.2.1 (page 96)	**Separating DNA Samples on an Agarose Gel**	30 min – 2 h
3.1.2.2 (page 97)	**Transferring DNA to a Membrane (Southern Blot)** Stage A: Depurinating and Denaturing DNA Stage B: Capillary transfer of DNA to nylon membrane Stage C: Fixing DNA to membrane by UV or baking	1 h overnight 3 min – 2 h
3.1.2.3 (page 99)	**Prehybridizing the Blot with DIG Easy Hyb**	30 min
3.1.2.4 (page 100)	**Hybridizing the DIG-labeled DNA Probe to DNA on the Blot** Stage A: Incubate Blot Membrane with Labeled Probe[1] Stage B: Wash Membrane Free of Unhybridized Probe	4 – 16 h 45 min

[1] The procedures for this stage describe the use of a DIG-labeled DNA probe to detect DNA on a blot. However, you may also use a DIG-labeled oligonucleotide probe to detect DNA targets if you make certain modifications to Procedures 3.1.2.1–3.1.2.4. See "How To Use DIG-labeled Oligonucleotide Probes To Detect DNA Targets on a Southern Blot" on page 102 for details.

This icon means you can stop the protocol at the end of the procedure and store the product for a time before starting the next procedure.

3.1.2.1. Separating DNA Samples on an Agarose Gel

Step	Action	Time
1	Prepare a suitable electrophoresis gel in a buffer such as TBE. Make the gel as thin as possible. *For optimal results, do not put ethidium bromide into the gel or the running buffer. Ethidium can cause uneven background problems if the gel is not run long enough.*	
2	Load small amounts of the target DNA samples (after mixing them with a suitable DNA loading buffer). Generally, keep the target DNA concentration **low**. For example, we recommend that you load: ▶ 2.5 –5 µg of human genomic DNA per lane, or ▶ Up to 10 µg of genomic DNA per lane if the genome (*e.g.* plant DNA) is more complex than human DNA, or ▶ <1 ng of plasmid DNA per lane.	

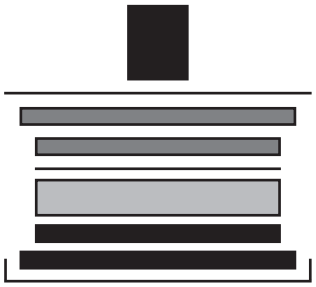
▶▶▶

Step	Action	Time
3	As a molecular weight marker, load approx. 5 µl of a DIG-labeled DNA Molecular Weight Marker (mixed with DNA loading buffer) per lane. *The amount needed will depend on the expected size of your hybridization product. Make sure to load enough marker to produce prominent bands that are about the same size as your hybrid.*	
4	Run the gel until the DNA bands are well separated	30 min – 2 h
5	To assess the quality of the target DNA, stain the gel briefly in 0.25 – 0.50 µg/ml ethidium bromide. Examine the gel under UV light.	15 – 30 min

3.1.2.2. Transferring DNA to a Membrane (Southern Blot, Capillary Transfer Method)

Step	Action	Time
1	Do one of the following: ▶ If your hybridization target is 5 kb or less, go to Step 3. ▶ If your hybridization target is >5 kb, go to Step 2.	
2	To depurinate the DNA prior to transfer, do the following: ▶ Submerge gel in 250 mM HCl, with shaking at room temperature, until the bromophenol blue marker changes from blue to yellow. *Do not exceed 10 min if you are treating human DNA.* *Do not exceed 20 min if you are treating plant DNA.* ▶ Rinse the gel with sterile, double distilled water. *Note: Depurination is only necessary if the target DNA is >5 kb.*	10–20 min
3	To denature the DNA in the gel, do the following: ▶ Submerge the gel in Denaturation Solution (0.5 M NaOH, 1.5 M NaCl) for 2 × 15 min at room temperature, with gentle shaking. ▶ Rinse the gel with sterile, double distilled water.	30 min
4	Submerge the gel in Neutralization Solution (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl) for 2 × 15 minutes at room temperature.	30 min
5	Equilibrate the gel for at least 10 min in 20× SSC.	10 min

3

Step	Action	Time
6	Set up the blot transfer as follows, avoiding the formation of air bubbles: ▶ Place a piece of Whatman 3MM paper that has been soaked with 20× SSC atop a "bridge" that rests in a shallow reservoir of 20× SSC. ▶ Place the gel atop the soaked sheet of Whatman 3MM paper. Roll a sterile pipette over the sandwich to remove all air bubbles that formed between the gel and paper. ▶ Cut a piece of Positively Charged Nylon Membrane to the size of the gel. ▶ Place the dry membrane on the DNA-containing surface of the gel. Use a pipette to eliminate air bubbles as above. ▶ Complete the blot assembly by adding a dry sheet of Whatman 3MM paper, a stack of paper towels, a glass plate, and a 200 – 500 g weight. The finished blot transfer "sandwich" should look like this:  Weight (200 - 500 g) Glass plate Stack of paper towels Whatman 3 MM paper, dry Nylon Membrane Agarose gel Whatman 3 MM paper, soaked Bridge, resting in a reservoir of 20× SSC	15 min
7	Allow the blot to transfer overnight in Transfer Buffer (20× SSC).	ON
8	While the blot is still damp, fix the DNA to the blot by either of the following methods: **IF you want to use...** / **THEN...** UV crosslinking: ▶ Place the membrane (DNA side facing up) on Whatman 3MM paper that has been soaked in 2× SSC. ▶ Expose the wet membrane to UV light, according to standard protocols, *e.g.* 1 – 3 min in a transilluminator.[1] ▶ Rinse the membrane briefly in sterile, double distilled water. ▶ Allow membrane to air dry. Baking: ▶ Wash the membrane briefly in 2× SSC.[2] ▶ Bake the membrane either: ▶ at 120°C for 30 min, or ▶ at 80°C for 2 h. [1] We use a UV Stratalinker (at 120 mJ) to immobilize DNA. You can also use a UV transilluminator, but you need to determine the exposure time experimentally. Generally, you can crosslink with short wave UV in 1 min or less. For long wave UV, try 2 - 3 min. Some transilluminators will require up to 5 min irradiation. [2] Do not let 20× SSC (transfer buffer) bake on the membrane. The dry salt crystals will give "starlike" signals during the chemiluminescent detection assay.	3 min – 2 h
9	Do one of the following: **IF you want to...** / **THEN...** Continue the procedure: Use the membrane immediately in Procedure 3.1.2.3. Stop now and store the blot for later use: Store the dry blot (between two sheets of Whatman 3MM paper in a sealed bag) at 4°C.	

3.1.2.3. Prehybridizing the Blot with DIG Easy Hyb

- *You can also use Procedures 3.1.2.3 and 3.1.2.4 to detect DNA on a dot blot.*
- *Do not allow the membrane to dry at any time during prehybridization, hybridization or detection (Section 4 in this chapter). If the membrane dries or sticks to a second membrane (e.g., during simultaneous processing of blots), the assay will have a high background.*
  Exception: *The membranes can be air dried after the last high stringency wash and stored at 4°C for later analysis. However, a stored membrane cannot later be stripped and reprobed.*

Step	Action	Time
1	Determine the appropriate hybridization temperature (T_{hyb}) according to the characteristics of your probe, target, and hybridization buffer. Use the formula on page 162 of Chapter 5 A to calculate T_{hyb}. ***Example****: If you are using DIG Easy Hyb and your target is mammalian DNA containing 40% GC sequences, the optimal hybridization temperature (T_{hyb}) should be 42°C.* *T_{hyb} is a stringent hybridization temperature for probes that are 80%–100% homologous to target. If the degree of homology of your probe and target is < 80%, you should lower the T_{hyb} (i.e., reduce the calculated T_{hyb} a further 1.4°C for each additional 1% mismatch).*	2 – 3 min
2	▶ Determine how much DIG Easy Hyb you will need for the procedures below. For every 100 cm^2 (*e.g.*, 10 cm × 10 cm) of membrane, you will need: ▶ 10 ml of DIG Easy Hyb for the prehybridization step, and ▶ 3.5 ml of DIG Easy Hyb for the hybridization step. ▶ Place the correct amount of DIG Easy Hyb in a sterile plastic tube, then place the tube in a water bath set at the correct hybridization temperature (see Step 1 above).	2 – 3 min
3	Place the blot into a Hybridization Bag (Cat. No. 11 666 649 001). *Prehybridization and hybridization steps can also be performed in almost any container that can be tightly sealed, such as temperature resistant plastic or glass boxes, petri dishes, roller bottles, or sealable plastic bags. The container must be sealed during the procedure to prevent the hybridization buffer from releasing NH_4 and changing the pH of the incubations.*	2 – 3 min
4	Heat seal closely around the blot, to reduce the dimensions of the bag and minimize the amount of prehybridization solution, hybridization solution, and probe needed.	2 – 3 min
5	▶ Add the appropriate amount of prewarmed DIG Easy Hyb to the bag (10 ml per 100 cm^2). ***Tip:*** You need enough buffer in the bag so that the bag looks slightly puffy when sealed. ▶ If you are using a Roche hybridization bag, remove the spigot (cut it off!) and seal the bag all around the blot, while removing air bubbles.	2 – 3 min
6	▶ Place the hybridization bag flat on the bottom of a rotating water bath. ▶ Place weights on the corners of the bag (but not touching the blot) to secure it. ▶ Incubate the blot for at least 30 minutes at the correct hybridization temperature (see Step 1). Agitate the membrane gently during this prehybridization step.	30 min

▶▶▶

Step	Action	Time
7	Do one of the following: **IF you...** / **THEN...** Want to continue → Use the membrane immediately in Procedure 3.1.2.4. Want to wait a while before continuing → Leave the membrane agitating in DIG Easy Hyb for up to several hours. This will not affect the result.	

3.1.2.4. Hybridizing the DIG-labeled DNA Probe to DNA on the Blot

Step	Action	Time
1	From your estimate of the amount of labeled probe you made (determined in Section 2.2.2.2 or Section 2.5.2.2), determine how much DIG-labeled probe you need for hybridization. ***Tip*:** If you are detecting single-copy genes in human genomic DNA, we recommend using at least 25 ng random primed labeled probe, or 2 µl PCR-labeled probe per ml hybridization buffer.	1 min
2	Prepare the hybridization solution as follows: ▶ Withdraw the appropriate amount of labeled probe from the tube containing the product of the labeling reaction. ▶ Place the labeled probe into a microcentrifuge tube along with 50 µl of water. ▶ Place the microcentrifuge tube into a boiling water bath for 5 minutes to denature the probe. ▶ Chill the probe quickly in an ice bath. ▶ Immediately add the denatured probe to a tube containing the appropriate amount of prewarmed DIG Easy Hyb (3.5 ml per 100 cm^2) and mix by inversion to form the hybridization solution.	10 min
3	Add the hybridization solution to the blot as follows: ▶ Cut open the sealed hybridization bag and pour out the prehybridization buffer. ▶ **Immediately** add hybridization solution containing DIG-labeled probe to the bag. ▶ Seal the bag all around the blot while removing air bubbles.	2 - 3 min
4	▶ Place the hybridization bag flat on the bottom of a rotating water bath. ▶ Place weights on the corners of the bag (but not touching the blot) to secure it. ▶ Incubate blot with probe for 6–16 h at the appropriate hybridization temperature (see Step 1 of Procedure 3.1.2.3). Agitate the blot gently during the incubation. ***Tip*:** We recommend overnight hybridization. However, if high sensitivity is not required, the incubation can be shortened. Most hybridizations will be complete after 6 h.	6 - 16 h

Step	Action	Time
5	▶ Add 200 ml Low Stringency Buffer (2× SSC containing 0.1% SDS) to a plastic tray. *In this and the following steps, the amount of buffer depends upon the size of the tray you are using. For each step, be sure the membrane is completely covered with solution.* ▶ At the end of the incubation, cut open the bag and pour off the hybridization solution from the blot. ***Tip*:** This hybridization solution can be saved in a tube at –20°C for future use and can be reused 3 – 5 times. ▶ **Immediately** place the membrane in a plastic tray with the Low Stringency Buffer.	2 min
6	▶ Incubate the tray at room temperature for 5 min with shaking. ▶ Pour off the used buffer, and immediately add 200 ml fresh Low Stringency Buffer. ▶ Incubate the tray an additional 5 min at room temperature with shaking.	10 min
7	During Step 6, preheat the High Stringency Buffer to the correct wash temperature in a shaking water bath. ***Tip*:** The correct High Stringency Buffer and wash temperature depend on the probe and target. For example, if you are detecting human or other mammalian DNA, use the following guidelines:	5 min
8	▶ Pour off the used Low Stringency Buffer. ▶ **Immediately** add the preheated High Stringency Buffer to the tray containing the blot. ▶ Incubate the blot twice (2×15 min) with preheated High Stringency Buffer at the correct wash temperature (as determined in Step 7). Shake the blot gently during the washes.	30 min

For a probe with the following...		Use the following High Stringency	
Homology to Target	**GC-content**	**Buffer**	**Temperature**
80 – 100%	Average (40%)	0.5× SSC + 0.1% SDS	65°C, if probe is >100 bp <65°C[1], if probe is short (100 bp or less)
<80%	Average (40%)	0.5× SSC + 0.1% SDS	Approx. 60°C[1]
80 – 100%	High (50%)	0.1× SSC + 0.1% SDS	68°C

[1] Exact temperature must be determined empirically.

3.1.2.5. What To Do Next

IF you want to...	THEN...
See the results of your hybridization	Go to Section 4, "Techniques for Detection of Hybridization Probes on a Blot" on page 115 of this chapter.
Store the blot for later use	Air dry the membrane and store it in a sealed bag at 4°C for later detection. *Stored, dry membranes cannot be stripped and reprobed after the detection assay.*
Learn how to optimize DNA hybridization protocol	Go to Section 3.3 "Getting the Best Results from Blots" on page 112 of this chapter.
See examples of results obtained with DIG-labeled DNA probes	Go to Section 4.1.4, "Typical Results with the Chemiluminescent Detection Assay" on page 121 of this chapter. Go to Section 4.2.3, "Typical Results with the Chromogenic Detection Assay" on page 127 of this chapter.

3.1.3 How To Use DIG-labeled Oligonucleotide Probes To Detect DNA Targets on a Southern Blot

If you want to use a DIG-labeled oligonucleotide probe instead of a DIG-labeled DNA probe to detect DNA on a Southern blot, you must modify Procedures 3.1.2.1 – 3.1.2.4 in the following ways:

When using oligonucleotide probes instead of DNA probes, change this parameter...	In the following way	Step(s) affected
Amount of target DNA loaded on gel	Increase the amount of DNA in each gel sample to: ▶ 10 µg human genomic DNA, or ▶ 2 ng plasmid DNA	▶ Procedure 3.1.2.1, Step 2
Temperature used for prehybridization, hybridization, and High Stringency Wash	▶ Decrease temperature to: (Tm of oligonucleotide) –10°C [see Note 1]	▶ Procedure 3.1.2.3, Steps 1, 2 and 6 ▶ Procedure 3.1.2.4, Steps 4, 7, and 8
Amount of labeled probe used in hybridization	Change the probe concentration (per ml hybridization buffer) to: ▶ 1–10 pmol of 3' end-labeled oligo, or ▶ 0.1–10 pmol of tailed oligo [see Note 2]	▶ Procedure 3.1.2.4, Step 1
Hybridization time	▶ Reduce the incubation time to 1 – 6 h	▶ Procedure 3.1.2.4, Step 4

[1] T_m of oligonucleotide = [(# G + C residues) × 4°C] + [(# A + T residues) × 2°C]

[2] When using tailed oligos, add 0.1 mg/ml poly(A) and 5 µg/ml poly(dA) to the hybridization buffer to prevent nonspecific binding of tails to membrane.

3.2 Hybridization of RNA Probes to a Northern Blot

A DIG-labeled RNA probe is ideal for detecting RNA on a Northern blot. RNA probes offer much better sensitivity for detecting low amounts of RNA target than DNA probes.

DNA probes can also be used to detect RNA targets if the samples on the blot contain a large amount of RNA. For more information, see "How To Use DIG-labeled DNA Probes To Detect RNA Targets on a Northern Blot" in Section 3.2.3 (page 111).

This section describes how to separate total RNA electrophoretically, transfer the RNA to a nylon membrane (Northern blot), and use DIG-labeled RNA probes to detect a unique mRNA on the blot. The RNA hybridization probe for this procedure can be prepared by the transcriptional labeling procedure (Section 2.3, page 74 of this chapter). Topics in this section include:

3.2.1 Materials Required for Electrophoresis, Blotting, and Hybridization

You will need the following reagents and equipment for the procedures below:

Reagent/Equipment	Description/Product
For all procedures	
Autoclaved, DMDC- or DEPC-treated water	▶ For preparation of all reagents needed in procedures
Oven, 200°C	▶ For baking glassware to ensure inactivation of RNases
RNase ZAP	▶ For decontaminating lab benches, labware
Powder-free gloves, sterile forceps	▶ For RNase-free handling of lab supplies, membrane
20× SSC	▶ 3 M NaCl; 300 mM sodium citrate, pH 7.0 (Cat. No. 11 666 681 001) (For preparing wash and transfer buffers)
For electrophoresis of RNA	
Agarose, nucleic acid grade	▶ See Chapter 2, page 48 for ordering information on suitable agaroses

▶▶▶

Reagent/Equipment	Description/Product
MOPS buffer, 10× conc.	▶ 200 mM MOPS, 50 mM sodium acetate, 20 mM EDTA, pH 7.0
Loading buffer	▶ MOPS/deionized formamide/formaldehyde/glycerol/ Bromophenol Blue (see procedure for details)
DIG-labeled RNA Molecular Weight Marker	▶ Molecular weight marker which can be visualized with anti-DIG antibody. For available DIG-labeled markers, see ordering information on page 48.
For northern blot transfer/prehybridization/hybridization	
Labeled RNA probe	▶ Prepared by transcriptional labeling (Section 2.3, page 74 of this chapter)
Nylon Membranes, Positively Charged	▶ Cat. No. 11 417 240 001 (0.3 × 0.3 m roll) ▶ Cat. No. 11 209 272 001 (20 × 30 cm) ▶ Cat. No. 11 209 299 001 (10 × 15 cm)
Whatman 3MM paper	▶ For capillary transfer
UV transilluminator or UV crosslinker	▶ To fix RNA to nylon membrane
Water bath, 68°C	▶ For prewarming prehybridization and hybridization buffers
Shaking water bath, 68°C	▶ For prehybridization/hybridization incubations, stringent washes
DIG Easy Hyb Granules (Hybridization buffer)	▶ Cat. No. 11 796 895 001, or ▶ Already included in the DIG Northern Starter Kit, Cat. No. 12 039 672 001 (see Section 2.3 for details) *Reconstituted Easy Hyb buffer (from granules) is stable at room temperature for 1 month.*
Hybridization Bags	▶ Cat. No. 11 666 649 001
Plastic or glass incubation trays	▶ For stringent washes
Low stringency buffer	▶ 2× SSC, containing 0.1% SDS
High stringency buffer	▶ 0.1× SSC, containing 0.1% SDS

3.2.2 Procedures

It is extremely important to use only RNase-free glassware, plasticware, and solutions for all these procedures.

The RNA hybridization protocol involves the procedures listed in the flow chart below. An estimate of the time required for each procedure is also given in the chart.

break	3.2.2.1 (page 106)	**Separating RNA Samples on an Agarose Gel**	2 h – overnight
break	3.2.2.2 (page 107)	**Transferring RNA to a Membrane (northern blot)** Stage A: Capillary transfer of RNA to nylon membrane Stage B: Fixing RNA to membrane by UV or baking	8 h – overnight 3 min – 2 h
	3.2.2.3 (page 108)	**Prehybridizing the Blot with DIG Easy Hyb**	30 min
break	3.2.2.4 (page 110)	**Hybridizing the DIG-labeled RNA Probe to RNA on the Blot** Stage A: Incubate Blot Membrane with Labeled Probe1 Stage B: Wash Membrane Free of Unhybridized Probe	6 – 16 h 45 min

[1] The procedures for this stage describe the use of a DIG-labeled RNA probe to detect RNA on a blot. However, you may also use a DIG-labeled DNA probe to detect RNA targets if you make certain modifications to Procedures 3.2.2.1–3.2.2.4. See "How To Use DIG-labeled DNA Probes To Detect RNA Targets on a Northern Blot" on page 111 for details.

break *This icon means you can stop the protocol at the end of the procedure and store the product for a time before starting the next procedure.*

3

3.2.2.1. Separating RNA Samples on an Agarose Gel

There are many protocols for separating RNA on gels. We recommend using a formaldehyde/MOPS gel, because we have found it to be easy to handle and reliable.

Do not include ethidium bromide in the gel. For more information and recommendations on gel electrophoresis and blot transfer, see page 28, Chapter 2.

Step	Action	Time
1	Prepare the gel reagents as follows: **Solution** / **Composition** 10× MOPS, pH 7.0: ▶ 200 mM MOPS buffer; ▶ 50 mM Sodium acetate; ▶ 20 mM EDTA; ▶ Adjust pH to 7.0 with NaOH Running Buffer[1]: 1× MOPS Loading Buffer[2]: ▶ 250 µl of 100% Deionized formamide; ▶ 83 µl of 37% Formaldehyde; ▶ 50 µl 10x MOPS; ▶ 50 µl 100% RNase-free Glycerol *Either purchase RNase-free glycerol or omit glycerol entirely from the buffer. Glycerol cannot be autoclaved! See Step 8 for information on running samples that do not contain glycerol.* ▶ 10 µl 2.5% Bromophenol blue; ▶ 57 µl DMDC- or DEPC-treated water 1 Always autoclave the MOPS running buffer before use. It is a common myth that you should not autoclave MOPS, because it will turn yellow and will not work. Our MOPS buffers are always yellow, and always work. 2 Always make the working solution of the loading buffer fresh just before mixing it with samples.	–
2	Prepare a 1% × 2% agarose gel (depending on the size of the RNA to be separated) in 1× MOPS buffer containing 2% formaldehyde. ***Example:*** *Melt 1.53.0 g agarose in a mixture of 141.9 ml 1× MOPS buffer and 8.1 ml 37% formaldehyde. Pour gel.*	30 min
3	Mix 2 volumes freshly made Loading Buffer with 1 volume of each RNA sample containing either: ▶ 1 µg total RNA, or ▶ 100 ng mRNA. *Recommended for using DIG-labeled RNA probes, when using DNA probes, please see page 11 (3.2.3.).*	1 min
4	As a molecular weight marker, load approx. 5 µl of a DIG-labeled RNA Molecular Weight Marker (mixed with loading buffer) per lane. *The amount needed will depend on the expected size of your hybridization product. Make sure to load enough marker to pro duce prominent bands that are about the same size as your hybrid.*	–
5	Denature the RNA/Loading Buffer mixtures at 65°C for 10 min.	10 min
6	Immediately chill the denatured samples on ice for 1 min.	1 min

▶▶▶

Step	Action	Time
7	Do one of the following: ▶ If your loading buffer contains glycerol, load the samples on the gel, cover the gel with running buffer, then go to Step 9. ▶ If your loading buffer does not contain glycerol, go to Step 8.	–
8	(Optional) If you omitted glycerol from your loading buffer to avoid potential RNase contamination, do the following: ▶ Load the samples onto a dry gel. ▶ Fill the slot carefully with running buffer. ▶ Add running buffer only up to the top of the gel (but not over the sample slots). ▶ Run the gel at a higher voltage (*i.e.*, >34 V/cm) for about 10 min to get the samples into the gel. ▶ Add extra running buffer to submerge the gel completely.	10 min
9	Run the gel at 34 V/cm for at least 2 h (preferably overnight), until the RNAs are well separated.	2 h over night
10	To assess the quality of the target RNA after electrophoresis, stain the gel briefly in 0.25 – 0.50 µg/ml ethidium bromide. Examine the gel under UV light.	15 – 30 min

■

3.2.2.2. Transferring RNA to a Membrane (Northern Blot, Capillary Transfer Method)

We do not recommend alkali treatment of the RNA before transfer, because it can easily destroy the target RNA. We have been able to transfer an 11.5 kb mRNA without alkali treatment, so it seems to us the treatment is not necessary.

Step	Action	Time
1	Soak the gel twice (2 × 15 min) in 20× SSC to remove formaldehyde (which can inhibit transfer).	30 min
2	Set up a blot transfer as follows, avoiding the formation of air bubbles between any two parts of the blot "sandwich": ▶ Place a piece of Whatman 3MM paper that has been soaked with 20× SSC atop a "bridge" that rests in a shallow reservoir of 20× SSC. ▶ Place gel, facing down, on top of the soaked sheet of Whatman 3MM paper. Roll a sterile pipette over the sandwich to remove all air bubbles that formed between the gel and paper. ▶ Cut a piece of Positively Charged Nylon Membrane to the size of the gel. ▶ Place the dry membrane carefully on top of the gel. Use a pipette to eliminate air bubbles as above. ▶ Complete the blot assembly by adding two dry sheets of Whatman 3MM paper, cut to the size of the gel, a stack of paper towels, a glass plate, and a 200 – 500 g weight. *See Step 6 of Procedure 3.1.2.2, page 98 in this chapter, for a diagram of the complete blot "sandwich".*	15 min
3	Let the RNA transfer (at least 6 h, preferably overnight) under RNase-free conditions, with sterile, RNase-free 20× SSC as transfer buffer.	6 h - ON
4	The next day, disassemble the transfer stack.	

▶▶▶

Step	Action	Time
5	While the membrane is still damp, fix the RNA to the blot by either of the following methods: **IF you want to use...** → **THEN...** UV crosslinking → ▶ Place the membrane (RNA side facing up) on Whatman 3MM paper that has been soaked in 2× SSC. ▶ Expose the wet membrane to UV light, according to standard protocols, *e.g.* 1 – 3 min in a transilluminator.[1] ▶ Rinse the membrane briefly in sterile, double distilled water. ▶ Allow membrane to air dry. Baking → ▶ Wash the membrane briefly in 2x SSC to remove excess salt.[2] ▶ Bake the membrane either: ▶ at 120°C for 30 min, or ▶ at 80°C for 2 h. [1] We use a UV Stratalinker (at 120 mJ) to immobilize RNA. You can also use a UV transilluminator, but you need to determine the exposure time experimentally. Gen erally, you can crosslink with short wave UV in 1 min or less. For long wave UV, try 2 – 3 min. Some transilluminators will require up to 5 min irradiation. [2] Do not let 20 SSC (transfer buffer) bake on the membrane. The dry salt crystals will give "star-like" signals during the chemiluminescent detection assay.	3 min – 2 h
6	Do one of the following: **IF you want to...** → **THEN...** Continue the procedure → Use the membrane immediately in Procedure 3.2.2.3 Stop now and store the membrane for later use → Store the dry blot (between two sheets of Whatman 3MM paper in a sealed bag) at 4°C.	–

3.2.2.3. Prehybridizing the Blot with DIG Easy Hyb

▶ *You can also use Procedures 3.2.2.3 and 3.2.2.4 to detect RNA on a dot blot.*

▶ *Do not allow the membrane to dry at any time during prehybridization, hybridization or detection (Section 4 in this chapter). If the membrane dries or sticks to a second membrane (e.g., during simultaneous processing of blots), the assay will have a high background.* ***Exception:*** *The membranes can be air dried after the last high stringency wash and stored at 4°C for later analysis. However, a stored membrane cannot later be stripped and reprobed.*

Step	Action	Time
1	Reconstitute the DIG Easy Hyb Granules by adding 64 ml of sterile, double-distilled, DMDC- or DEPC-treated water to the plastic bottle. Stir for 5 min at 37°C to dissolve the granules.	5 min
2	Determine how much DIG Easy Hyb you will need for the procedures below. You will need enough buffer to completely cover the membrane during incubations. ***Example:*** *If you use a hybridization bag, for every 100 cm^2 (e.g., 10 cm x 10 cm) of membrane, you will need:* ▶ 10 – 15 ml of DIG Easy Hyb for the prehybridization step, and ▶ 3.5 ml of DIG Easy Hyb for the hybridization step.	2 – 3 min
3	Place the correct amount of DIG Easy Hyb (calculated in Step 2 above) in a sterile tube, then place the tube in a water bath set at 68°C. ***Tip:*** For most RNA:RNA hybridizations, the correct temperature for prehybridization and prehybridization in DIG Easy Hyb will be 68°C. However, if you use a heterologous RNA probe, you may have to alter the prehybridization/hybridization temperature.	2 – 3 min
4	Place the blot into a Hybridization Bag (Cat. No. 11 666 649 001). *Prehybridization and hybridization steps can also be performed in almost any container that can be tightly sealed, such as temperature resistant plastic or glass boxes, petri dishes, roller bottles, or sealable plastic bags. The container must be sealed during the procedure to prevent the hybridization buffer from releasing NH_4 and changing the pH of the incubations.*	2 – 3 min
5	▶ Add the appropriate amount of prewarmed DIG Easy Hyb to the bag (10 – 15 ml per 100 cm^2). ***Tip:*** You need enough buffer in the bag so that the bag looks slightly puffy when sealed. ▶ If you are using a Roche Hybridization Bag, remove the spigot (cut it off!) and seal the bag all around the blot, while removing air bubbles.	2 – 3 min
6	▶ Place the Hybridization Bag flat on the bottom of the rotating water bath. ▶ Place weights on the corners of the bag (but not touching the membrane) to secure it. ▶ Incubate the blot for 30 minutes at 68°C. Agitate the membrane gently during this prehybridization step.	30 min
7	Do one of the following:	

IF you...	THEN...
Want to continue	Use membrane immediately in Procedure 3.2.2.4.
Want to wait a while before continuing	Leave the membrane agitating in DIG Easy Hyb for up to several hours. This will not affect the result.

3

3.2.2.4. Hybridizing the DIG-labeled RNA Probe to RNA on the Blot

Step	Action	Time
1	During the prehybridization incubation (Step 6 of Procedure 3.2.2.3), prepare the hybridization solution as follows: ▶ Determine how much DIG-labeled RNA probe you need. ***Tip*:** The recommended concentration for transcriptionally labeled RNA probe is 100 ng probe per ml hybridization buffer. ▶ Withdraw the appropriate amount of labeled probe from the tube containing the product of the labeling reaction. ▶ Place the labeled probe into a microcentrifuge tube along with 50 µl RNase-free, double distilled water. ▶ Place the microcentrifuge tube into a boiling water bath for 5 minutes to denature the probe. ▶ Chill the probe quickly in an ice bath. ▶ Centrifuge the tube briefly. ▶ Immediately add the denatured probe to a tube containing the appropriate amount of prewarmed DIG Easy Hyb (3.5 ml per 100 cm^2) and mix by inversion to form the hybridization solution.	10 min
2	Add the hybridization solution to the blot as follows: ▶ Cut open the sealed hybridization bag and pour out the prehybridization buffer. ▶ Immediately replace with prewarmed hybridization solution containing DIG-labeled probe. ▶ Seal the bag all around the blot while removing air bubbles.	2 – 3 min
3	▶ Place the hybridization bag flat on the bottom of a shaking water bath at 68°C. ▶ Place weights on the corners of the bag (but not touching the membrane) to secure it. ▶ Incubate blot with probe at 68°C for 6 – 16 h, with gentle agitation. ***Tip:*** We recommend overnight hybridization. However, if high sensitivity is not required, the incubation can be shortened. Most hybridizations will be complete after 6 h.	6 – 16 h
4	After the hybridization is complete: ▶ Fill an RNase-free plastic container (tray, dish, etc.) with enough Low Stringency Buffer (2× SSC containing 0.1% SDS) to completely cover the membrane. ▶ Cut open the bag and pour off the hybridization solution from the blot. ▶ **Immediately** submerge the membrane in the tray containing Low Stringency Buffer.	2 min
5	▶ Incubate the tray at room temperature for 5 min with shaking. ▶ Pour off the used buffer and immediately cover the membrane with fresh Low Stringency Buffer. ▶ Incubate the tray an additional 5 min at room temperature with shaking.	10 min
6	During the incubations in Step 5, preheat High Stringency Buffer (0.1× SSC containing 0.1% SDS) to 68°C.	–
7	▶ Pour off the used Low Stringency Buffer. ▶ **Immediately** add the preheated High Stringency Buffer to the tray containing the blot. ▶ Incubate the blot twice (2 × 15 min, with shaking) in High Stringency Buffer at 68°C. *If your probe is <80% homologous to the target RNA, you should perform this wash step at a lower temperature (determined empirically).*	30 min

3.2.2.5. What To Do Next

IF you want to...	THEN...
See the results of your hybridization	Go to Section 4, "Techniques for Detection of Hybridization Probes on a Blot," page 115 of this chapter.
Learn how to optimize RNA hybridization protocol	Go to Section 3.3 "Getting the Best Results from Blots," page 112 of this chapter.
See examples of results obtained with DIG-labeled RNA probes	Go to Section 4.1.4, "Typical Results with the Chemiluminescent Detection Assay," page 121 of this chapter.

3.2.3 How To Use DIG-labeled DNA Probes To Detect RNA Targets on a Northern Blot

If you want to use a DIG-labeled DNA probe instead of a DIG-labeled RNA probe to detect RNA targets, you must modify Procedures 3.2.2.1 – 3.2.2.4 in the following ways:

When using DNA probes instead of RNA probes, change this parameter...	In the following way	Step(s) affected
Amount of target RNA loaded on gel	Increase the amount of RNA in each gel sample to: ▶ 5 µg total RNA, or ▶ 500 ng mRNA	▶ Procedure 3.2.2.1, Step 3
Temperature used for prehybridization, hybridization, and high stringency wash	▶ Decrease temperature to 50°C	▶ Procedure 3.2.2.3, Steps 3 and 6 ▶ Procedure 3.2.2.4, Steps 3, 6, and 7
Amount of labeled probe used in hybridization	▶ Change the labeled probe concentration to 20 – 50 ng DIG-labeled DNA probe/ml hybridization buffer	▶ Procedure 3.2.2.4, Step 1

3

3.3 Getting the Best Results from Blots

3.3.1 Critical Hints about Electrophoresis, Blotting, and Hybridization

Technique	Hints
Electrophoresis	▶ Avoid loading high amounts of target nucleic acid on the gel. If there is too much target on the gel, the probe may detect minor degradation products. ▶ When separating target nucleic acids on agarose, do not include ethidium bromide in the gel. Ethidium can cause uneven background problems if the gel is not run long enough.
Blotting	▶ When touching the nylon blot membrane, always wear gloves and handle the membrane only at the edges, with forceps. ▶ When setting up the blot "sandwich", make sure the dry blotting paper and paper towels on top of the membrane do not touch the gel or buffer-saturated paper and sponge under the gel. Place Parafilm around the gel to prevent any such contact. ▶ The weight used in the blot "sandwich" should vary according to the size of the gel. We suggest using only a 200 g weight for mini-gels and increasing the weight to a maximum of 500 g (for 20 × 20 cm gels). ▶ During the overnight blot transfer, do not let the stack of the paper towels at the top of the "sandwich" become completely saturated. If necessary, remove the wet paper towels and replace them with dry towels.
Prehybridization	▶ Use enough buffer to completely cover the membrane during prehybridization and hybridization incubations. The amounts needed will depend on the shape and capacity of the container you use for the incubations. ▶ You may want to process multiple blots in a single container. If you do, make sure there is enough liquid in the container to keep the blots from sticking together during the incubations. They should move freely when agitated. ▶ Do not allow the membrane to dry at any time from the beginning of prehybridization through probe-hybrid visualization (Section 4 in this chapter). If the membrane dries or sticks to a second membrane, the assay will have a high background.

▶▶▶

Technique	Hints
Hybridization	▶ The correct temperature for most hybridizations in DIG Easy Hyb will be 37° – 42°C (for DNA:DNA), 68°C (for RNA:RNA), or 50°C (for DNA:RNA). However, if your probe has a high GC content (> 50%) or imperfect (< 80%) homology to thetarget, you may have to alter the temperature. For more information, see page 163 of Chapter 5 A. ▶ To prevent membrane from drying, do not pour off prehybridization buffer until hybridization solution containing probe has been prepared. ▶ Always prewarm hybridization buffer before adding it to the membrane. Prewarming prevents reannealing of probe secondary structures and nonspecific binding of probe to membrane. ▶ The concentration of probe is critical to the success of the hybridization. Use the gel electrophoresis method (Section 2.2.2.2; for PCR-labeled probes only) to accurately estimate the concentration of labeled probe. Then you will know how much probe to add to the hybridization buffer. ▶ Always heat denature the probe (68°C, 10 min) before using it. (Do not boil!) Even single-stranded probes have secondary structures, which need to be disrupted for optimal hybridization to occur. ▶ Heat sealing closely around the blot minimizes the amount of prehybridization solution, hybridization solution, and probe needed. ▶ DIG-labeled probes are stable for at least 1 year. If stored in hybridization buffer, DIG-labeled DNA probes may even be reused several (generally 3 – 5) times. However, we do not recommend storing or reusing hybridization solutions containing RNA probes, because RNA probes are inherently unstable and difficult to keep free of RNase.
Post-hybridization washes	▶ To prevent membrane from drying out during the washes, do not pour off the used solution from one step until the new solution for the next step has been prepared. ▶ If your DNA probe is < 80% homologous to the target nucleic acid, you should perform the high stringency wash step at a lower temperature (*e.g.* 60°C) than recommended in the procedure (Procedure 3.1.2.4, Step 7). ▶ If your DNA probe has a high GC content (50% or more), use 0.1× SSC + 0.1% SDS as the High Stringency Buffer and increase high stringency wash temperature to 68°C ▶ Always preheat High Stringency Buffer before use. Otherwise, the buffer will not reach the correct temperature during the high stringency wash.

3

3.3.2 Troubleshooting the Hybridization Blot

Problem ☹	Possible Cause 😐	Recommendation ☺
Low amount of nucleic acid on membrane	Inefficient capillary transfer	▶ After blot transfer, stain gel with ethidium bromide to verify efficient transfer of nucleic acids. ▶ See "Critical Hints" about blotting above.
	Target nucleic acid degraded	▶ Check the amount and integrity of target DNA or RNA. ▶ When preparing/using RNA, maintain strict RNase-free conditions at all times. ▶ Repeat blot with freshly prepared target DNA or RNA.
Low probe sensitivity	Inefficient probe labeling	▶ Follow **all** recommendations in Section 2 for preparation of template. ▶ See the troubleshooting guides in Sections 2.1 – 2.3 for suggested remedies.
	Target degraded	▶ Prepare a new template.
	Probe concentration too low	▶ Increase probe concentration. (Do not exceed 100 ng DIG-labeled RNA or 25 ng DIG-labeled DNA/ml hybridization buffer.) ▶ Allow hybridization to continue overnight.
	Problems with detection assay	▶ See troubleshooting guide for detection assay (Section 4.1.3 in this chapter).
High background on blot	Target concentration in sample too high	▶ If using DNA probes, do not use more than 5 µg human genomic DNA, 10 µg plant genomic DNA, or 1 ng plasmid DNA on a Southern blot. ▶ If using oligo probes, do not use more than 10 µg human genomic DNA or 2 ng plasmid DNA on a Southern blot. ▶ If using RNA probes, do not use more than 1 mg of total RNA or 100 ng of mRNA per lane on a Northern blot. ▶ If using DNA probes, do not use more than 5 µg total RNA or 500 ng mRNA on a Northern blot.
	Probe concentration too high	▶ Decrease probe concentration (see maximum concentration guidelines under "Low sensitivity" above). ▶ Determine optimal probe concentration experimentally.
	Wrong type of membrane	▶ Use positively-charged nylon membranes from Roche, which are tested for the DIG-System.
	Membrane dried during procedure	▶ Do not allow the membrane to dry at any stage of the prehybridization, hybridization, or detection procedures. ▶ Make sure membrane is completely covered by solutions at all times.
	Ineffective stringency washes	▶ Check temperature of stringency washes. ▶ Always prewarm wash solution to correct temperature before using. ▶ For Northern blots, increase temperature of High Stringency Wash to 70°C.
	Other possible causes	▶ See detection troubleshooting guide (Section 4.1.3) in this chapter. ▶ See Chapter 5 B for different background problems and their remedies.

4. Techniques for Detection of Hybridization Probes on a Blot

After you finish the hybridization procedure (Section 3), use one of the methods in this section to detect the probe-target hybrids. The methods described in this section include:

For information on this topic	Turn to Section	Starting on page
Chemiluminescent Methods for Detection of Probes on a Blot ***Key Products:*** ready-to-use CSPD and CDP-*Star*	4.1	115
Chromogenic Methods for Detection of Probes on a Blot ***Key Product:*** NBT/BCIP Stock Solution,	4.2	123

4.1 Chemiluminescent Methods for Detection of Probes on a Blot

The most sensitive method for detecting probe-target hybrids involves an alkaline phosphatase-conjugated anti-DIG antibody and chemiluminescent (light-generating) alkaline phosphatase substrates. This section describes how to use the chemiluminescent alkaline phosphatase substrates, CSPD and CDP-*Star* to visualize the probe-target hybrids on a Southern or Northern blot. Topics in this section include:

3

4.1.1 Materials Required for Chemiluminescent Detection

Reagent	Description/Product
Membrane washing and blocking buffers	▶ DIG Wash and Block Buffer Set (DNase and RNase-free), Cat. No. 11 585 762 001
Anti-Digoxigenin-alkaline phosphatase antibody	▶ Available separately as Cat. No. 11 093 274 910 ▶ Already included in the DIG High Prime DNA Labeling and Detection Starter Kit II, Cat. No. 11 585 614 910 ▶ Already included in the DIG Northern Starter Kit, Cat. No. 12 039 672 910
Chemiluminescent alkaline phosphatase substrate choose either:	
▶ Ready-to-use CSPD	▶ Available separately as Cat. No. 11 755 633 001 ▶ Already included in the DIG High Prime DNA Labeling and Detection Starter Kit II, Cat. No. 11 585 614 910
▶ Ready-to-use CDP-*Star*	▶ Available separately as Cat. No. 12 041 677 001 ▶ Already included in the DIG Northern Starter Kit, Cat. No. 12 039 672 910
Lumi-Film Chemiluminescent Detection Film	▶ Cat. No. 11 666 657 001 ▶ X-ray film, optimized for chemiluminescent visualization

4.1.2 Procedure

The chemiluminescent detection procedure and the time required for each stage are listed in the flow chart below.

	Visualizing Probe-Target Hybrids by Chemiluminescent Assay	
4.1.2.1 (page 117)	Stage A: Wash and block the membrane	35 min
	Stage B: Localize the probe-target hybrids with anti-DIG	30 min
	Stage C: Wash unbound antibody off membrane	30 min
	Stage D: Detect DIG on blot with chemiluminescence	1045 min

4.1.2.1. Visualizing Probe-Target Hybrids by Chemiluminescent Assay

Before you start: First prepare the working solutions for the detection procedure.

Working Solution	Composition/Preparation	Storage/Stability
Maleic Acid Buffer[1]	0.1 M Maleic acid, 0.15 M NaCl; adjust with NaOH (solid) to pH 7.5	room temperature, stable
Blocking Solution[2]	Dilute 10× Blocking Solution (vial 6) 1:10 with Maleic Acid Buffer	Prepare fresh
Washing buffer[1]	0.1 M Maleic acid, 0.15 M NaCl; pH 7.5; 0.3% (v/v) Tween 20	room temperature, stable
Antibody Solution[2]	Centrifuge Anti-Digoxigenin-AP for 5 min at 10 000 rpm in the original vial prior to each use, and pipet the necessary amount carefully from the surface. Dilute Anti-Digoxigenin-AP 1:10 000 (75 mU/ml) in Blocking solution.	Prepare fresh (2 h, 4°C)
Detection Buffer[1]	0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20°C)	room temperature, stable

1 Concentrated (10×) stock solutions of these reagents are available in the DIG Wash and Block Buffer Set. Unless otherwise indicated, dilute them tenfold with sterile, double-distilled water to prepare the working solutions. For detection of RNA, use only DMDC- or DEPC-treated water.

2 Stock (undiluted) reagent available as vial 4 in DIG-High Prime Starter Kit II or vial 5 in DIG Northern Starter Kit. Also available separately as a powder.

- *Unless otherwise indicated, all of the following incubations are performed at room temperature with shaking.*
- *The volumes suggested below are for a 10 cm × 10 cm (100 cm²) blot processed in a plastic tray. If you are processing smaller blots (e.g. membrane discs for colony/plaque hybridization) or using a smaller container, you can use smaller volumes. Be sure each solution completely covers the membrane and keeps it from sticking to the container as it is shaken. As an alternative to Steps 6 – 9 of this procedure, you may use the transparency technique (described in Section 6.1.2.3, page 138 of this chapter), which conserves chemiluminescent substrate.*

Step	Action	Time
1	▶ Transfer the membrane to a plastic container (*e.g.*, a tray) containing 100 ml Washing Buffer. ▶ Incubate for 2 min at room temperature, with shaking. ▶ Discard the Washing Buffer.	2 min
2	▶ Add 100 ml Blocking Solution to tray. ▶ Incubate membrane for 30 min, with shaking. ***Tip:*** This blocking step can last up to 3 hours without affecting results. ▶ Discard the Blocking Solution.	30 min
3	▶ Add 20 ml Antibody Solution to the tray. ▶ Incubate the membrane for 30 min, with shaking. ▶ Discard the Antibody Solution.	30 min
4	Wash membrane twice (2 × 15 min) with 100 ml portions of Washing Buffer.	2 × 15 min
5	Equilibrate membrane 3 min in 20 ml Detection Buffer.	3 min

▶▶▶

Step	Action	Time
6	Using gloves, add the chemiluminescent substrate to the blot, as follows: ▶ Place the membrane (DNA/RNA side facing up) inside a development folder (acetate sheets), hybridization bag, or other tightly sealable envelope-like container. Do **not** wrap membrane in plastic wrap, which cannot be tightly sealed. ▶ For every 100 cm^2 of membrane, apply 1 ml (20 – 30 drops) Ready-to-use CSPD or CDP-*Star*, dropwise, over the surface of the blot until the entire surface is evenly soaked. ▶ **As you are applying the substrate, immediately** cover the dampened part of the membrane with the second side of the container so the substrate is spread evenly over the membrane. Do not let air bubbles form between the membrane and the upper surface of the container.	2 min
7	▶ Incubate membrane for 5 min at room temperature. ▶ Squeeze excess liquid out of the container and seal the sides of the container close to the membrane.	5 min
8	Do one of the following: ▶ If you are using CSPD, incubate damp membrane (in the sealed container) for 10 min at 37°C to enhance the luminescence reaction. ▶ If you are using CDP-*Star*, skip this step and go to Step 9.	0 – 10 min
9	Expose the sealed envelope (containing the membrane) at room temperature to: ▶ Lumi-Film X-ray film (15 – 25 min)	5 – 25 min
10	Based on the result in Step 9, adjust the exposure time to get a darker or lighter band pattern. ***Tip:*** Repeat exposures can be made up to two days after the addition of substrate.	

4.1.2.2. What To Do Next

IF you want to...	THEN...
See typical results achieved with the chemiluminescent detection method	See Section 4.1.4 on page 121 of this chapter.
Strip your probe off the membrane so you can hybridize another probe to the membrane	Go to Section 5, "Techniques for Stripping and Reprobing a Membrane," on page 128 of this chapter.
Store the membrane for later use	Go to Section 5, "Techniques for Stripping and Reprobing a Membrane," on page 128 of this chapter.

4.1.3 Troubleshooting the Chemiluminescent Detection Procedure

Problem ☹	Possible cause 😐	Recommendation ☺
Low sensitivity	Inefficient probe labeling	▶ Check labeling efficiency of your DIG-labeled probe by the gel electrophoresis method (Procedure 2.2.2.2, Chapter 3; for PCR-labeled probes only). ▶ For suggested remedies, see the troubleshooting guides for labeling in Sections 2.1 – 2.3.
	Concentration of labeled probe too low	▶ Increase probe concentration (Do not exceed 100 ng labeled RNA probes or 25 ng labeled DNA probe/ml hybridization buffer). ▶ Allow hybridization to continue overnight.
	Wrong type of membrane	▶ We recommend positively charged nylon membranes, from Roche . Other types of nylon membranes, *e.g.* Biodyne A (Pall), are also suitable but need longer exposure times to X-ray fi lm. ▶ Nitrocellulose membranes cannot be used in chemiluminescent detection procedures.
	Inefficient hybridization	▶ Increase the concentration of DIG-labeled probe in the hybridization solution (see above for maximum allowable probe concentration). ▶ Check hybridization temperature.
	Insufficient preincubation (with CSPD only)	▶ Preincubate the membrane (Procedure 4.1.2.1, Step 8) for a longer time (> 30 min, up to 12 h) before exposure to X-ray film.
	Insufficient exposure time	▶ Increase time of exposure to film. ▶ The type of film may also influence the sensitivity. We have tested and can recommend Kodak XAR and DuPont Cronex 4.
High background	Inefficient labeling	▶ Purify template by phenol/chloroform extraction and/or ethanol precipitation before labeling. ▶ Do not use EDTA in the buffers used for storage of template. ▶ Make sure that the probe does not contain crosshybridizing vector sequences.
	Concentration of labeled probe too high *This is the most common cause of high background.*	▶ Decrease concentration of DIG-labeled probe. (Do not use more than 100 ng labeled RNA probe or 25 ng labeled DNA probe/ml hybridization buffer). ▶ Determine optimal probe concentration experimentally. *The critical probe concentration limit (to eliminate background) can be determined by hybridizing increasing amounts of probe to a membrane that contains no target nucleic acid. (See "How To Optimize Single Copy Gene Detection Easily" on page 176 in Chapter 5 B.)*
	Target concentration in sample too high	▶ Do not exceed the amounts of target nucleic acids suggested in Sections 3.1.2.1 and 3.2.2.1.
	Ineffective stringency washes	▶ Always prewarm wash solution to correct temperature before using. ▶ Check temperature of stringency washes. ▶ **For Northern blots only:** If necessary, increase temperature of high stringency wash to 70°C.

▶▶▶

Problem ☹	Possible cause 😐	Recommendation ☺
High background	Wrong type of membrane	▶ Use our function-tested positively charged nylon membranes. *Although the protocol is optimized for the use of positively charged nylon membranes, some positively charged membranes from other vendors are very highly charged and can cause background (see Section 5.1 of Chapter 2 for more information).* ▶ Lot-to-lot variations in some membranes may also cause problems.
	Membrane dried during procedure	▶ Do not permit the membrane to dry during the prehybridization, hybridization, or detection procedures. ▶ Make sure the membrane is completely covered with liquid in each step. The size of the tray used in the procedures will affect the amount of buffer needed during prehybridization, hybridization, and detection steps. ▶ In each step, minimize the time between pouring off one solution and adding the next. This will prevent drying of the membrane during the detection procedure. ▶ Cover the membrane as you add chemiluminescent substrate to prevent the membrane from drying. ▶ Do not use plastic wrap for chemiluminescent substrate step. Plastic wrap cannot be sealed and allows the membrane to dry. It also allows substrate to be trapped in its creases, causing high background in these areas. Use only transparent containers that can be tightly sealed.
	Antibody concentration too high	▶ Decrease concentration of anti-DIG-AP conjugate. ▶ Increase volume of the washing and blocking solution and duration of the washing and blocking steps. ▶ Before use, centrifuge anti-DIG-AP conjugate to remove any precipitates that may have formed in the preparation. ▶ Do not incubate the membrane with antibody for longer than 30 min.
	Excessive preincubation (with CSPD only)	▶ Shorten the time you preincubate membrane before X-ray exposure (Procedure 4.1.2.1, Step 8).
	Excessive exposure	▶ Shorten exposure time to X-ray film The signal intensity increases with time.
Spotty background	Antibody contained high molecular weight “complexes”	▶ Always centrifuge antibody stock solution for 5 min at full speed before taking aliquot (of supernatant) to make Antibody Solution.
	SSC crystals	▶ Wash membrane briefly in 2× SSC before fixation by baking.
	Protein contamination in probe	▶ Filter labeled probe through a 0.45 µm cellulose acetate filter (*e.g.,* from Schleicher and Schuell). *Do not use nitrocellulose filters; the probe will bind to these filters.* ▶ Purify probe with the High Pure PCR Product Purification Kit.
Specific, but unwanted, hybridization bands on Northern blots	SP6, T3 or T7 sequences in transcription plasmid that are complementary to ribosomal RNA	▶ Reduce amount of target RNA. When using DIG-labeled RNA probes, do not use more than 1 µg of total RNA or 100 ng of mRNA per lane. ▶ Prepare mRNA from your total RNA and use the purified mRNA as target.

4.1.4 Typical Results with the Chemiluminescent Detection Assay

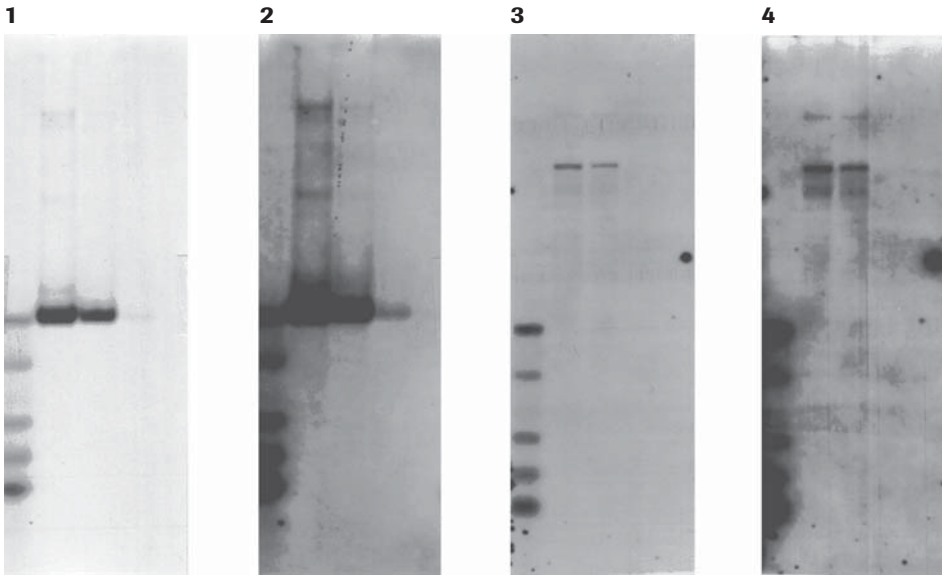


Figure 15. Detection of a Rare Human mRNA with a DIG-labeled RNA Probe. Two identical Northern blots were prepared. The samples on each were various amounts of total human skeletal RNA (lane 2, 100 ng; lane 3, 50 ng; lane 4, 5 ng; and lane 5, 1 ng). A DIG-labeled RNA Molecular Weight Marker (lane 1) was included on each gel. The blot on the left (panels 1, 2) was probed with a DIG-labeled antisense RNA probe complementary to the highly expressed actin mRNA. The blot on the right (panels 3, 4) was probed with a DIG-labeled antisense RNA probe complementary to the rare mRNA for the transcription factor CTF1. Hybrids were visualized with a chemiluminescent assay and exposure of the blot to X-ray film for 15 min (panels 1 and 3) or 90 min (panels 2 and 4).

Result and conclusion: DIG-labeled RNA probes could detect the highly expressed actin mRNA in as little as 5 ng total RNA and the rare CTF1 mRNA in as little as 50 ng of total RNA. In both cases, a 15 min exposure was sufficient to detect the target; the 90 min exposure did not increase the sensitivity of the assay. Thus, DIG-labeled RNA probes can easily detect a rare mRNA in total RNA. An assay with a radioactively labeled RNA probe (not shown) required overnight exposure to detect the CTF1 mRNA in over 10 µg of total RNA!

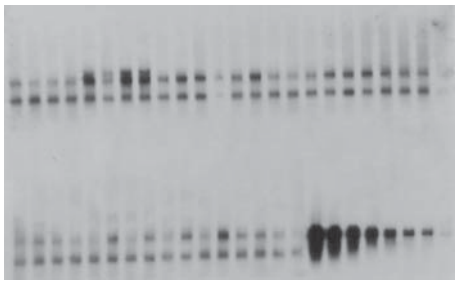


Figure 16. Detection of NGF mRNA on a Northern Blot with Simultaneous Detection of an Internal Control RNA. A Northern blot screening system was developed to screen rat hippocampi for expression of nerve growth factor (NGF) mRNA. The samples contained purified hippocampus mRNA from rats that had been exposed to different experimental conditions. The samples were denatured and separated on a glyoxal gel, transferred to a blot membrane, and probed with a DIG-labeled antisense NGF RNA probe in hybridization buffer containing 50% formamide. To ensure that equal amounts of mRNA were loaded from each animal, the RNA samples were "spiked" with 5 pg of a truncated sense transcript of the NGF gene (recovery standard) before preparation of mRNA. This internal control will always generate a hybridization signal with the NGF probe (lower band in each lane). This enables the results to be normalized across samples without requiring a separate hybridization reaction to a reference "housekeeping" gene. In addition, the blot contains an *in vitro* transcribed, full-length NGF sense transcript (last 8 lanes, lower right) as a calibration standard. Blotting and chemiluminescent detection were done according to standard DIG System techniques. (Data kindly provided by Bastian Hengerer, Ciba Geigy, Switzerland)

Result: The calibration sample shows that the probe can easily detect full-length NGF mRNA in as little as 0.6 pg of target mRNA (last lane, lower right).

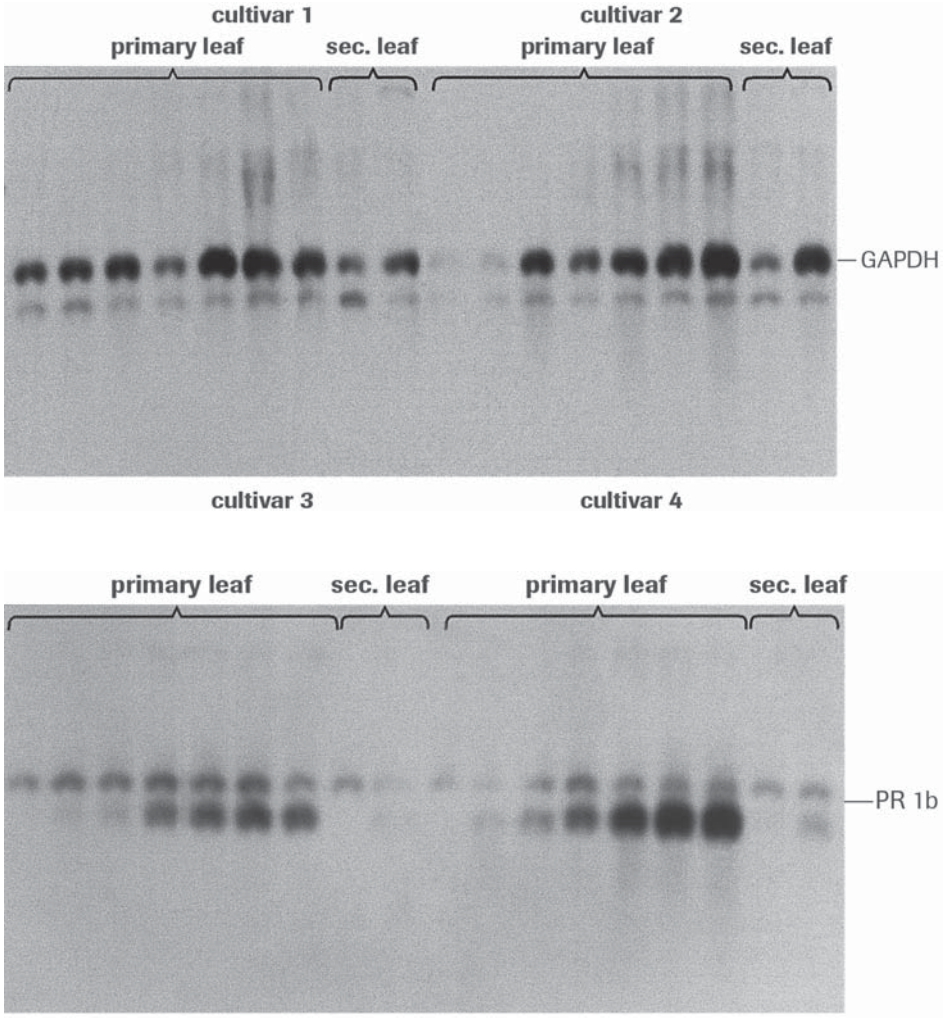


Figure 17. Probing a Northern Blot with Two Probes without Stripping between Hybridizations. A study of unique transcript accumulation during senescence of barley leaves required screening many samples (primary and secondary leaves of different cultivars). To simplify the study, one of our customers devised a method for probing a single Northern blot with two labeled RNA probes, without an intermediate stripping step. One µg samples of total RNA were used as targets. First, a DIG-labeled antisense RNA probe (A) was used to detect a constitutive mRNA (GAPDH) as a control. Next, a fluorescein-labeled probe (B) was used to detect a single-copy gene that accumulates during the senescence process (PR 1b). The probe concentration in each experiment was 100 ng per ml of DIG Easy Hyb. Standard hybridization and stringent washing conditions were used. The hybrids were detected with alkaline phosphatase-labeled anti-DIG (A) and anti-fluorescein (B). The signal from each probe was obtained with CDP-*Star* and a 10 min exposure to X-ray film. (Data courtesy of C. Peterhänsel, University of Aachen, Germany.)

Result and conclusion: Because two different labels (DIG-UTP and fluorescein-UTP) were used to make the two probes, they could be used in consecutive hybridization reactions with no intermediate stripping required. The anti-DIG antibody used in the first detection was denatured during the second hybridiza-tion and therefore did not interfere with the second assay. There was no loss of sensitivity with the second probe, since both assays required the same exposure (10 min).

This system was much faster and more sensitive than a radioactive assay. The comparable assay (result not shown) with radioactive (^{32}P-labeled) probes required a 36 h exposure to X-ray film to detect the GAPDH and PR 1b mRNAs in 10 µg total RNA.

4.2 Chromogenic Methods for Detection of Probes on a Blot

Because they offer both high sensitivity and speed, most investigators use the chemiluminescent procedures described in Sections 4.1 of this chapter for visualization of DIG-labeled probes and their targets. However, you may prefer to use chromogenic detection methods, since they offer the following advantages:

- **Chromogenic signals are visible without exposure to film**
  Useful in situations where the specialized equipment required for recording chemiluminescent signals are not available.
- **Different color substrates are available**
  Two or more substrates may be used in combination to detect more than one target (see Figure 19 for an example).

For these methods, Roche offers several chromogenic alkaline phosphatase substrates (see table below).

For a complete listing of all chromogenic and colorimetric substrates available from Roche, please see our general biochemical catalog.

Chromogenic Alkaline Phosphatase Substrates that May be Used with the DIG System

Product	Applications	Color Produced[1]	Incubation Time Required[2]	Comments
NBT/BCIP Stock Solution (Cat. No. 11 681 451 001) **NBT/BCIP Ready-to-use Tablets** (Cat. No. 11 697 471 001)	▶ Southern or western blots; colony and plaque lifts; in situ assays	▶ Blue, insoluble	5 min – 16 h	▶ May be used with both nitrocellulose and nylon membranes ▶ Signal can be renewed by rehydrating blot
HNPP Solution[3]	▶ Southern or Northern blots; colony and plaque lifts	▶ Red, insoluble	2 – 16 h	▶ Component of HNPP Fluorescent Detection Set

[1] The precipitate formed by all these substrates is visible to the n ked eye. A photocopy or photograph of the result may be made as a permanent record.

[2] Incubation time depends greatly on the amount of hybrid present and the complexity of the signal.

[3] HNPP solution is not available as a separate reagent, but is sold as part of the HNPP Fluorescent Detection Set (Cat. No. 11 758 888 001). The set is used for fluorescent ELISA assays, but the HNPP solution in the set may be used as a stand-alone chromogenic reagent.

This section gives detailed procedures for using NBT/BCIP to detect DIG-labeled probes on a blot. Topics in this section include:

3

4.2.1 Materials Required for Chromogenic Detection

Reagent	Description
Membrane washing and blocking buffers	▶ DIG Wash and Block Buffer Set (DNase and RNase-free), Cat. No. 11 585 762 001
Anti-Dioxigenin-alkaline phosphatase antibody	▶ Available separately as Cat. No. 11 093 274 910 ▶ Already included in the DIG High Prime DNA Labeling and Detection Starter Kit II, Cat. No. 11 585 614 910 ▶ Already included in the DIG Northern Starter Kit, Cat. No. 12 039 672 910
Chromogenic alkaline phosphatase substrate ▶ NBT/BCIP	▶ Available separately as Cat. No. 11 681 451 001 (solution) or 11 697 471 001 (tablets) (see table p. 123) ▶ Included in the DIG High Prime DNA Labeling and Detection Starter Kit I, Cat. No. 11 745 832 910
Detection Buffer	▶ 0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20°)
TE buffer	▶ 10 mM Tris, 1 mM EDTA, pH approx. 8

4.2.2 Procedures

The chromogenic detection procedures and the time required for each stage are listed in the flow chart below.

4.2.2.1 (page 125)	**Localizing Probe-Target Hybrids with Anti-DIG** Stage A: Wash and block the membrane Stage B: Localize the probe-target hybrids with anti-DIG Stage C: Wash unbound antibody off membrane	35 min 30 min 30 min
4.2.2.2 (page 126)	Stage D1: Visualizing Probe-Target Hybrids with NBT/BCIP	10 – 16 h

4.2.2.1. Localizing the Probe-Target Hybrids with Anti-DIG

- *Unless otherwise indicated, all of the following incubations are performed at room temperature with shaking.*
- *The volumes below are for a 10 cm × 10 cm (100 cm²) blot processed in a plastic tray. If you are processing smaller blots (e.g. membrane discs for colony/plaque hybridization) or using a smaller container, you can use smaller volumes. Be sure each solution completely covers the membrane and keeps it from sticking to the container as it is shaken.*

Before you start: First prepare the working solutions for the detection procedure:

Working Solution	Composition/Preparation	Storage/Stability
Maleic Acid Buffer[1]	▶ 0.1 M Maleic acid, 0.15 M NaCl; adjust with NaOH (solid) to pH 7.5	room temperature, stable
Blocking Solution[2]	▶ Dilute 10× Blocking Solution (vial 6) 1:10 with Maleic Acid Buffer	Prepare fresh
Washing Buffer[1]	▶ 0.1 M Maleic acid, 0.15 M NaCl; pH 7.5; 0.3% (v/v) Tween 20	room temperature, stable
Antibody Solution[2]	▶ Centrifuge Anti-Digoxigenin-AP for 5 min at 10 000 rpm in the original vial prior to each use, and pipet the necessary amount carefully from the surface. Dilute Anti-Digoxigenin-AP 1: 5 000 (150 mU/ml) in Blocking solution.	Prepare fresh (2 h, 4°C)
Detection Buffer[1]	▶ 0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20°C)	room temperature, stable
TE buffer	▶ 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	room temperature, stable

[1] Concentrated (10×) stock solutions of these reagents are available in the DIG Wash and Block Buffer Set. Unless otherwise indicated, dilute them tenfold with sterile, double-distilled water to prepare the working solutions. For detection of RNA, use only DMDC- or DEPC-treated water.

[2] Note that the antibody dilution is different for the chromogenic and chemiluminescent detection procedures.

Step	Action	Time
1	▶ Transfer the membrane to a plastic container (*e.g.*, a tray) containing 100 ml Washing Buffer. ▶ Incubate for 2 min at room temperature, with shaking. ▶ Discard the Washing Buffer.	2 min
2	▶ Add 100 ml Blocking Solution. ▶ Incubate membrane for 30 min, with shaking. ***Tip:*** This blocking step can last up to 3 hours without affecting results. ▶ Discard the Blocking Solution.	30 min
3	▶ Add 20 ml Antibody Solution. ▶ Incubate the membrane for 30 min, with shaking. ▶ Discard the Antibody Solution.	30 min
4	Wash membrane twice (2 × 15 min) with 100 ml portions of Washing Buffer.	2 × 5 min
5	Equilibrate membrane 3 min in 20 ml Detection Buffer.	3 min
6	▶ For visualization with NBT/BCIP, go to Procedure 4.2.2.2.	

3

4.2.2.2. Visualizing Probe-Target Hybrids with NBT/BCIP

Step	Action	Time
1	Prepare the Color Substrate Solution by doing one of the following: **IF you are using...** / **THEN...** NBT/BCIP stock solution (Cat. No. 11 681 451 001) → Add 200 µl of the NBT/BCIP stock solution to 10 ml of Detection Buffer to make Color Substrate Solution[1]. NBT/BCIP tablets (Cat. No. 11 697 471 001) → Dissolve one NBT/BCIP tablet completely in 10 ml sterile, double distilled water to make Color Substrate Solution[1]. [1] Store the Color Substrate Solution protected from light.	1 – 3 min
2	▶ Cover **one** membrane completely with 10 ml Color Substrate Solution. *If you are working with more than one membrane, process each membrane separately.* ▶ Incubate the membrane in the dark **without shaking**. *The colored precipitate begins forming within a few minutes. The reaction is usually complete after 16 h.*	16 h
3	When the color reaction produces bands of the desired intensity, stop the reaction by rinsing the membrane for 5 min in 50 ml of TE buffer.	5 min
4	See Section 4.2.2.3, "What To Do Next".	

4.2.2.3. What To Do Next

IF you want to...	THEN...
Document the NBT/BCIP result (Procedure 4.2.2.2) and store the membrane	▶ Photocopy or photograph the wet membrane. ▶ Dry the membrane at room temperature and store the dry membrane at room temperature. *If you want to reprobe the membrane, do not let it dry. Instead, store it in TE buffer. (See below.) Colors may fade on the dry mem brane, but you can renew them by rewetting the disc with TE buffer.*
Strip your membrane so you can hybridize another probe to the membrane	▶ Store the membrane in TE buffer until you can strip it. ▶ Go to Section 5, "Techniques for Stripping and Reprobing a Membrane," on page 128 of this chapter.
See typical results achieved with the chromogenic detection methods	▶ See Section 4.2.3 below.

4.2.3 Typical Results with the Chromogenic Detection Assay

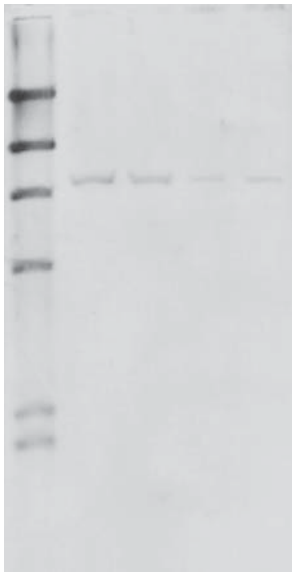


Figure 18. Detection of N-RAS in human buffy coat DNA. Various amounts of total DNA from the buffy coat fraction of human blood DNA were separated on a gel, then blot transferred to a membrane. The blot was hybridized to a PCR-labeled probe that recognizes the single copy N-RAS gene. Probe-target hybrids were visualized by a standard colorimetric DIG detection assay. The total amount of DNA in each sample is listed under the lanes on the blot. Lane 1 is a DIG-labeled DNA Molecular Weight Marker.

Result: The DIG-labeled probe, prepared by PCR, could detect the single copy N-RAS gene in as little as 0.25 µg total human buffy coat DNA on a Southern blot.

3

5. Techniques for Stripping and Reprobing a Membrane

If you want to remove (strip) a probe from the membrane so you can detect a second target with another probe, you may use the stripping and reprobing procedures included in this section.

5.1 Materials Required for Stripping Reactions

Reagent/Equipment	Description/Purpose
For stripping DIG-labeled DNA probe off membrane after any detection assay	
Stripping buffer	▶ 0.2 M NaOH, 0.1% SDS
2× SSC	▶ 0.3 M NaCl, 30 mM sodium citrate, pH 7.0
For decolorizing membrane after a colorimetric detection assay with NBT/BCIP	
Fume hood	▶ For decolorization incubation
Dimethylformamide	▶ For dissolving NBT/BCIP product
Large beaker	▶ For heating dimethylformamide
Heating plate	▶ For heating dimethylformamide
For stripping DIG-labeled RNA probe off membrane after a chemiluminescent detection assay	
Stripping buffer	▶ 50% deionized formamide; 5% SDS; 50 mM Tris-HCl, pH 7.5
2× SSC	▶ 0.3 M NaCl, 30 mM sodium citrate, pH 7.0

5.2 Procedures

If you let the membrane dry at any stage of the prehybridization, hybridization, or detection procedures, you cannot strip and reprobe that membrane.

The table below estimates the time required for each stripping procedure:

To remove this type of probe	After this type of assay	Requires approx.
DIG-labeled DNA probe	Chemiluminescent detection	30 – 40 min
DIG-labeled DNA probe	Chromogenic detection	approx. 2 h
DIG-labeled RNA probe	Chemiluminescent detection	1 – 2 h

5.2.1 Stripping DIG-labeled DNA Probe after Chemiluminescent Detection

Tip: The alkaline wash in this procedure will strip probes made with the alkali-labile DIG-dUTP supplied in most DIG labeling kits.

Step	Action	Performed with this Solution	Time
1	Rinse membrane thoroughly.	Double distilled water	1 min
2	Wash membrane twice at 37°C in Stripping Buffer.	0.2 M NaOH containing 0.1% SDS	2 × 15 min
3	Rinse membrane	2× SSC	5 min

5.2.2 Stripping DIG-labeled DNA Probe after Chromogenic Detection with NBT/BCIP

Step	Action	Performed with this Solution	Time
1	In a fume hood, heat a large beaker containing dimethylformamide to 50° – 60°C.	Dimethylformamide	–
2	Place the membrane disc in the heated dimethylformamide and incubate at 50° – 60°C until the blue color has been removed from the disc[1].	–	1 h
3	Rinse membrane thoroughly.	Double distilled water	1 min
4	Wash membrane twice at 37°C in Stripping Buffer.	0.2 M NaOH containing 0.1% SDS	2 × 20 min
5	Rinse membrane	2× SSC	5 min

[1] Changing the DMF occasionally will speed this procedure.

5.2.3 Stripping DIG-labeled RNA probe after Chemiluminescent Detection

Always prepare the stripping buffer just before use. With this technique, we have successfully stripped and reprobed a northern blot up to 24 times in our application lab.

Step	Action	Performed with this Solution	Time
1	Rinse membrane thoroughly.	Double distilled water	–
2	Wash membrane at 80°C in Stripping Buffer.	50% deionized formamide/ 5% SDS/50 mM Tris-HCl, pH 7.5	2 × 60 min
3	Rinse membrane thoroughly	2× SSC	5 min

5.2.4 What to Do Next

IF you want to...	THEN...
Reprobe membrane with a second DIG-labeled probe	Repeat the hybridization and detection procedure with a different DIG-labeled probe. *If you are reusing a stored hybridization solution, heat the solution to 68°C for 10 min (to denature the probe) before using it for hybridization.*
Store stripped membrane for later use.	Store the membrane wet in 2× SSC.

6. High Volume Screening Applications for DIG-labeled Probes

Section 3 of this chapter described how DIG-labeled probes could be used to find target nucleic acids on a single blot. This section describes how to use DIG-labeled probes to detect a few target nucleic acids in a large number of samples. The high volume methods described in this section may be used, for example, to screen genomic or cDNA libraries. These methods include:

6.1 Use of DIG-labeled Probes for Colony and Plaque Hybridization

This section describes how to use DIG-labeled DNA hybridization probes to screen bacterial colonies or phage plaques for complementary (recombinant) sequences.

Topics in this section include:

3

6.1.1 Materials Required for Colony/Plaque Hybridization

The complete screening protocol requires the following equipment, materials, and reagents. Many are available from Roche (catalog numbers given below).

Reagent / Equiment	Catalog Number	Description
For colony or plaque lifts		
Agar plates		▶ For display/support of colonies/plaques. *For plaques, we recommend using 0.7% Agarose MP (Cat. No. 11 388 983 001) in YT broth for top agar.*
Nylon Membranes for Colony and Plaque Hybridization	11 699 075 001 (82 mm discs) 11 699 083 001 (132 mm discs)	▶ Microporous, hydrophilic, neutral nylon membranes; ideal for colony or plaque lifts
Plastic film		▶ For equilibrating membranes with processing solutions
Denaturation solution		▶ 0.5 M NaOH, 1.5 M NaCl
Neutralization solution		▶ 1.5 M NaCl, 1.0 M Tris-HCl, pH 7.4 (21°C)
2× SSC		▶ 0.3 M NaCl, 30 mM sodium citrate, pH 7.0; tenfold dilution of 20× SSC (Cat. No. 11 666 681 001)
3MM paper (Whatman)		▶ For blotting nylon membranes
UV transilluminator or UV crosslinker		▶ To fix DNA to nylon membrane
For removing cellular debris (colony lifts only)		
Proteinase K solution, recombinant, PCR Grade	03 115 887 001 (1.25 ml) 03 115 828 001 (5 ml) 03 115 844 001 (25 ml)	▶ Enzyme solution, 10× conc., DNase-free
Incubator, set at 37°C		▶ For proteinase K incubation
For screening of colony/plaque lifts with a DIG-labeled probe		
DIG-labeled DNA probe, complementary to the sequence to be detected in the colony/plaque lifts		▶ DIG-labeled DNA probes may be prepared by random primed labeling, PCR, or other methods. We recommend using the PCR labeling method (as described in Section 2.2, page 64 of this chapter) for preparing the probes.
Boiling water bath		▶ For denaturing probe
DIG Easy Hyb	11 603 558 001	▶ Ready-to-use hybridization buffer; DNase- and RNase-free
Roller bottles or sealable bags, 275 ml capacity		▶ For prehybridization/hybridization incubations *Do not use open containers for incubations with DIG Easy Hyb.*
Incubator oven for roller bottles, set at 42°C		▶ For prehybridization/hybridization incubations
Low stringency buffer		▶ 2× SSC, 0.1% SDS
High stringency buffer		▶ 0.5× SSC, 0.1% SDS

▶▶▶

Reagent / Equiment	Catalog Number	Description
For immunochemical detection of probe-target hybrids		
DIG Wash and Block Buffer Set (DNase- and RNase-free)	11 585 762 001	▶ Washing buffer, 10× conc. ▶ Maleic Acid buffer for dilution of blocking solution, 10× conc. ▶ Blocking solution, 10× conc. ▶ Detection buffer, 10× conc.
Anti-Dioxigenin antibody, alkaline phosphatase (AP) conjugate	11 093 274 910	▶ Polyclonal antibody, Fab fragments, for detection of DIG in probe-target hybrids
Hybridization bags	11 666 649 001	▶ Plastic bags fitted with a spigot, which facilitates easy exchange of buffer solutions
For chemiluminescent visualization		
Chemiluminescent AP substrate – choose either:		
CSPD, 25 mM	11 655 884 001 (1 ml) 11 759 035 001 (2 × 1 ml) 11 759 043 001 (4 × 1 ml)	▶ 100× concentrated
CSPD, ready-to-use, 0.25 mM	11 755 633 001 (2 × 50 ml) 11 685 627 001 (1 ml)	▶ 1× concentrated ▶ 200 – 500× concentrated
CDP-*Star*, 25 mM	11 759 051 001 (2 × 1 ml)	
CDP-*Star*, ready-to-use, 0.25 mM	12 041 677 001 (2 × 50 ml)	▶ 2 – 5× concentrated
Lumi-Film Chemiluminescent Detection Film	11 666 657 001	▶ X-ray film, optimized for chemiluminescent visualization
3MM paper (Whatman)		▶ For removing excess liquid from membranes
For chromogenic visualization		
Chromogenic AP substrate – choose either:		
NBT/BCIP, made from:		
NBT solution	11 383 213 001	▶ 100 mg/ml 4-nitroblue tetrazolium chloride in 70% dimethylformamide
BCIP solution	11 383 221 001	▶ 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in dimethylformamide
TE buffer		▶ 10 mM Tris-HCl, 1 mM EDTA, pH 8.0; stop buffer for chromogenic reaction

3

6.1.2 Procedures

The procedures for colony and plaque screening and the time required for each are listed in the flow chart below. An estimate of the time required for each procedure is also given in the chart.

6.1.2.1 (page 134)	**Preparing Colony or Plaque Lifts**	1 - 2.5 h
6.1.2.2 (page 137)	**Hybridizing a DIG-labeled DNA Probe to the Colony/Plaque Lifts**	3.75 h
6.1.2.3 (page 138)	**Detecting Probe-Target Hybrids with a Chemiluminescent or Chromogenic Assay** Stage A: Wash and block membrane Stage B: Localize the probe-target hybrids with anti-DIG Stage C: Wash membrane	 35 min 30 min 30 min
6.1.2.3 (page 138)	Stage D1: Detect DIG on blot with CSPD	30 – 50 min
	or	
	Stage D2: Detect DIG on blot with CDP-Star	5 – 25 min
	or	
6.1.2.4 (page 139)	Stage D3: Detect DIG on blot with NBT/BCIP	10 – 16 h

6.1.2.1. Preparing Colony or Plaque Lifts

Step	Action	Time
1	Pre-cool agar plates containing colonies or plaques for approx. 30 min at 4°C. *For plaque lifts we recommend using our agarose-MP for top agar (0.7% in YT medium).*	30 min
2	While the plates are cooling, do the following: ▶ Place a sheet of thin plastic film (sometimes called “plastic foil”) on your bench. The size of the sheet depends on the number and size of membranes. *Do not use Saran Wrap because it tends to wrinkle.* ▶ For each membrane disc, place a 1 ml puddle (2 ml for 132 mm discs) of Denaturation Solution (0.5 M NaOH, 1.5 M NaCl) on the plastic film. *Be sure to leave enough space between the puddles so they don’t run together.* ▶ Cut three dry sheets of Whatman 3MM paper.	–

▶▶▶

Step	Action	Time
3	▶ Carefully place a membrane disc onto the surface of each precooled plate. Avoid air bubbles between membrane and agar. *Do not move the membrane once it rests on the plate.* ▶ Leave the membranes on the plates for approx. 1 min. *To be able to identify positive plaques or colonies on the plate, be sure to mark the orientation of the membrane relative to the plate.* ***Tip:*** For replicate lifts from a single plate, leave the second membrane on the plate for 2 min (rather than 1) to allow efficient transfer in spite of the reduced number of colonies/ plaques on the plate.	2 min
4	Use filter tweezers to: ▶ Remove each membrane disc carefully from the plates. ▶ Flip the disc so the colonies/plaque pattern is on top, and briefly blot the bottom of the membrane on a dry sheet of Whatman 3MM paper. ▶ Lower each membrane disc (with the colony/plaque side facing up) carefully onto one of the puddles on the plastic film (from Step 2). Make sure the membrane is completely equilibrated with the liquid. *Let the rim of the disc hit the drop first, then let the solution spread from there over the whole disc. If you place the center of the membrane disc on the puddle, it is difficult to get the solution distributed evenly on the disc.*	1 min
5	Incubate the discs for either: ▶ 5 min for plaques *Do not incubate plaques for longer than 5 min.* OR ▶ 15 min for colonies *Always incubate colonies the full 15 min.*	5 – 15 min
6	During the last 2 – –3 min of the incubation, prepare a new plastic film containing 1 ml (2 ml for 132 mm discs) puddles of the Neutralization Solution (1.5 M NaCl, 1.0 M Tris-HCl, pH 7.4).	–
7	After incubation is complete, place membrane discs on a new dry sheet of Whatman 3MM paper and let them air dry briefly.	1 min
8	▶ Discard the used plastic film. ▶ Transfer the discs (as in Step 4) to the new plastic film containing Neutralization Solution. ▶ Incubate for 5 or 15 min (as in Step 5).	5 – 15 min
9	During the last 2 – 3 min of the incubation, prepare a new plastic film containing 1 ml (2 ml for 132 mm discs) puddles of 2× SSC.	–
10	After the incubation is complete, repeat the air drying step (Step 7).	1 min
11	▶ Discard the used plastic film. ▶ Transfer the discs (as in Step 4) to the new plastic film containing 2× SSC. ▶ Incubate for 10 min.	10 min

▶▶▶

Step	Action	Time
12	Crosslink the transferred DNA to the disc by illuminating the disc with UV light for approx. 30 s to 5 min at a distance of 15 cm. *As an alternative to UV crosslinking, you may bake the DNA onto the membranes (at least 30 min at 80°C).* ***Tip:*** We use a UV Stratalinker (at 120 mJ) to immobilize DNA. You can also use a UV transilluminator, but you need to determine the exposure time experimentally. Generally, you can crosslink with short wave UV in 1 min or less. For long wave UV, try 2 – 3 min. Some transilluminators will require up to 5 min irradiation.	30 s – 5 min
13	Do one of the following: **IF you have prepared...** / **THEN...** Plaque lifts: Go to Procedure 6.1.2.2 (Hybridization). No further treatment of the membrane is necessary. Colony lifts: Go to Step 14 below.	
14	To remove cell debris (colony lifts only), treat the membrane with proteinase K as follows: ▶ Dilute proteinase K (>600 U/ml, 1422 mg/ml) 1:10 in 2× SSC. ▶ Place membrane disc on a square of aluminum foil or plastic film. ▶ For each 82 mm membrane disc, pipette 0.5 ml of diluted Proteinase K Solution onto the disc. *Use 1.0 ml diluted Proteinase K solution for a 132 mm disc.* ▶ Distribute the solution evenly and incubate the disc for 1 h at 37°C. Make sure that the ventilation of the incubator is turned off. ▶ Prewet a sheet of Whatman 3MM paper with sterile water and place the damp paper atop all the membrane discs. ▶ To press the paper firmly onto the membrane discs, draw a ruler or similar device over the surface of the paper. ▶ Gently lift the damp paper off the discs. *When the paper is lifted, all the colony debris should stick to it. If there is still debris left on the discs, repeat the blotting procedure with a fresh sheet of damp Whatman 3MM paper.* ▶ Once all debris has been removed, go to Procedure 6.1.2.2.	1.25 h

6.1.2.2. Hybridizing a DIG-labeled DNA Probe to the Colony/Plaque Lifts

Before you begin: For the procedure below, determine the appropriate hybridization temperature according to probe and target characteristics. If you are using DIG Easy Hyb as hybridization buffer, use the formulae below to calculate T_{hyb}:

- T_m of hybrid = 49.82 + 0.41[%G + C] – (600/L) [where % G+C = GC content of labeled probe, L = length (bp) of probe-target hybrid]
- T_{hyb} = 20° – 25°C below T_m of hybrid[where T_{hyb} = optimal hybridization temperature (in DIG-Easy Hyb)]

If you are using a different hybridization buffer , see Chapter 5 A for instructions on calculating T_{hyb}.

- ***Example:*** The procedure below assumes the hybridization buffer is DIG Easy Hyb, the target has a 50% GC content, and the probe is 100% homologous to target DNA. The T_{hyb} used is 42°C.

To avoid contamination and background formation, always use gloves and a pair of filter tweezers to handle the membrane discs.

Step[1]	Action	Performed with this Solution[1]	Time
1	Place up to 20 membrane discs (82 mm diameter) in a 275 ml roller bottle or sealable hybridization bag.	–	1 min
2	Prehybridize membranes at hybridization temperature (usually 42°C) in the closed container.	60 ml DIG Easy Hyb	1 h
3	Denature labeled probe (25 ng/ml) by boiling for 5 min at 95 – 100°C and rapidly place on ice.		5 min
4	Hybridize DIG-labeled DNA probe to membranes in the closed container at hybridization temperature.	▶ For 1 – 3 membranes: 6 ml of DIG Easy Hyb containing 25 ng DIG-labeled DNA probe/ml ▶ For 4 – 20 membranes: 10–15 ml of DIG Easy Hyb containing 25 ng DIG-labeled DNA probe/ml	2 h
5	Wash the membranes twice with Low Stringency Wash Buffer at RT.	2× SSC + 0.1% SDS	2 × 5 min
6	Wash the membranes twice with High Stringency Wash Buffer at 68°C.	0.5× SSC + 0.1% SDS *For E. coli DNA, use 0.1× SSC + 0.1% SDS at 68°C.*	2 × 15 min

[1] For details on these steps and solutions, see Section 3.1 on page 94 of this chapter.

3

6.1.2.3. Detecting Probe-Target Hybrids with a Chemiluminescent Assay (Transparency Technique)

Step	Action[1]	Performed with this Solution[1]	Time
1	Wash membrane briefly.	Washing Buffer	1 – 5 min
2	Block the membrane[2].	40 ml Blocking Solution	30 min
3	Let the antibody bind DIG label on the membrane.	15 ml of Dilute (1:10000) Antibody Solution	30 min
4	Wash the membrane twice to remove unbound antibody.	40 ml portions of Washing Buffer	2 x 15 min
5	Equilibrate the membrane.	20 ml Detection Buffer	2 – 5
6	Place the membrane on a sheet of plastic transparency film[3].	–	–
7	Cover the membrane with diluted chemiluminescent substrate in Detection Buffer.	For each 100 cm2 of membrane, use 500 µl of EITHER: ▶ 1:100 dilution of 25 mM stock CSPD, or ▶ undiluted 0.25 mM CSPD, ready-to-use, or ▶ 1:200 – 1:500 dilution of 25 mM stock CDP-*Star*, or ▶ 1:2 – 1:5 dilution of 0.25 mM CDP-*Star*, ready-to-use	5 min
8	Cover the damp membrane with a second sheet of transparency film.	–	–
9	Incubate for 5 min	CSPD or CDP-*Star*	5 min
10	Let excess liquid drip off membrane and heat seal the transparency "sandwich" around the damp membrane.	CSPD or CDP-*Star*	1 min
11	(Optional) Activate CSPD at 37°C.	CSPD only (**not** necessary with CDP-*Star*)	(10 min)
12	Expose the sealed bag (containing the membrane) to X-ray film	–	5 – 25 min

[1] For details on these steps and solutions, see Section 4.1 on page 115 of this chapter.

[2] The volumes given in Steps 2 – 5 are adequate for processing one 82 mm membrane disc in a small glass tray. For one 132 mm disc, double the volume stated in each step. If you are processing more than one disc, increase the volumes until there is enough liquid to completely cover the discs and keep them from sticking together.

[3] The transparency technique (described in Steps 6 – 12) conserves chemiluminescent substrate. These steps may also be performed in a hybridization bag if the amount of chemiluminescent substrate is doubled.

6.1.2.4. Detecting Probe-Target Hybrids with a Chromogenic Assay

Step	Action[1]	Performed with this Solution[1,2]	Time
	Perform Steps 1 – 5 of Procedure 6.1.2.3 above.	See Procedure 6.1.2.3, page 138	93 – 100 min
6	Cover membrane completely with Color Substrate Working Solution.	▶ 4 ml of fresh NBT/BCIP solution[3] in Detection Buffer	1 min
7	Incubate in the dark until hybridization signals are the desired intensity. *Do not move or shake the discs during this incubation.*	▶ NBT/BCIP (N/X)	10 – 16 h (for N/X)
8	Stop reaction by incubating membrane in TE buffer.	50 ml TE buffer	5 min
9	Document the result by photocopying or photographing the wet membrane[4].	–	–

[1] For details on these steps and solutions, see Section 4.2 on page 123 of this chapter.

[2] The volumes given are for processing one 82 mm membrane disc in a small glass tray. For one 132 mm disc, double the volume stated in each step. If you are processing more than one disc, increase the volumes until there is enough liquid to completely cover the discs and keep them from sticking together.

[3] Mix 34 µl NBT solution (100 mg/ml) and 35 µl BCIP solution (50 mg/ml) with 10 ml Detection Buffer.

[4] For long term storage, the membrane may be stripped of probe and stored damp in 2× SSC (see Section 5 on page 128 of this manual for details). Alternatively, bake the membrane disc at 80°C and store it dry at room temperature. If color fades (NBT/BCIP), rewet disc with TE buffer.

6.1.3 Critical Hints about Colony/Plaque Hybridization

Product	Description
Choice of membrane	For best results with the DIG System, use our specially developed Nylon Membranes for Colony and Plaque Hybridization. These membrane discs: ▶ Save time because they do not require prewetting ▶ Allow rapid, quantitative transfer and binding of DNA because of their 1.2 µm pores ▶ Are mechanically robust and have a high DNA binding capacity, allowing multiple rounds of stripping and rehybridization with different probes
Denaturation of colonies	Denature bacterial colonies for a full 15 min to ensure complete cell lysis.
Denaturation of plaques	For plaque lifts, limit the denaturation step to 5 min. Longer denaturation will lyse the bacterial lawn around the plaques and lead to high background.
Removal of colony cell debris	▶ Do not let the membrane dry during the proteinase K digestion. For instance, if the incubator used in this step has a vent fan, switch it off; the fan would dry the membrane instantly and the proteinase K would not be active on the dry membrane. ▶ If debris is not removed, the anti-DIG antibody may bind nonspecifically to denatured proteins in the debris, making it difficult to distinguish positive and negative colonies.

3

6.1.4 Typical Results of Colony/Plaque Screens with DIG-labeled Probes

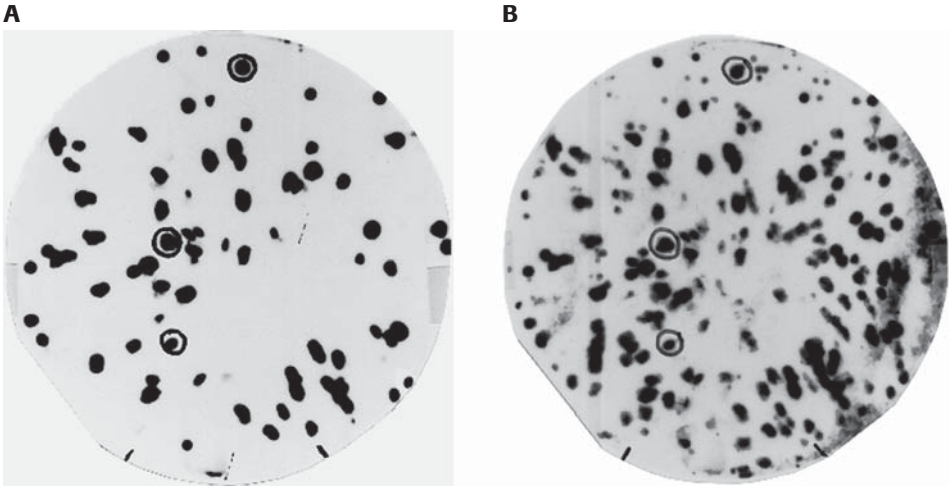


Figure 19. Screening Plaques Simultaneously with Two Different Probes. Two sets of M13 phage clones were prepared. The first set contained the gene for ampicillin resistance (Amp^r); the second contained the gene for tetracycline resistance (Tet^r). Aliquots of these two sets were mixed 1:1 and plated on a lawn of *E. coli*. A routine plaque lift was performed. The plaque lift was screened with a mixture of two probes, each present at 25 ng/ml, in DIG Easy Hyb. The first probe was a DIG-labeled probe complementary to the Amp^r gene; the second was a biotin-labeled probe complementary to the Tet^r gene. Probe-target hybrids were detected in two stages, using different antibodies, and finally visualized chemiluminescently with CSPD (45 s exposures). Between the two stages, the membrane was **not stripped** of probe and alkaline phosphatase (AP) from the first antibody **was not inactivated** with 50 mM EDTA.

Result: Panel A shows the result of the first stage detection with an anti-DIG-AP antibody. The visible plaques are Amp^r clones. Panel B shows the result of the second stage detection with streptavidin-AP, which recognizes the biotinylated probe. Since the membrane was not stripped or the AP inactivated between the stages, the visible plaques in panel B are a mixture of both Amp^r and Tet^r clones. The Tet^r clones can be identified by comparing panel B with panel A.

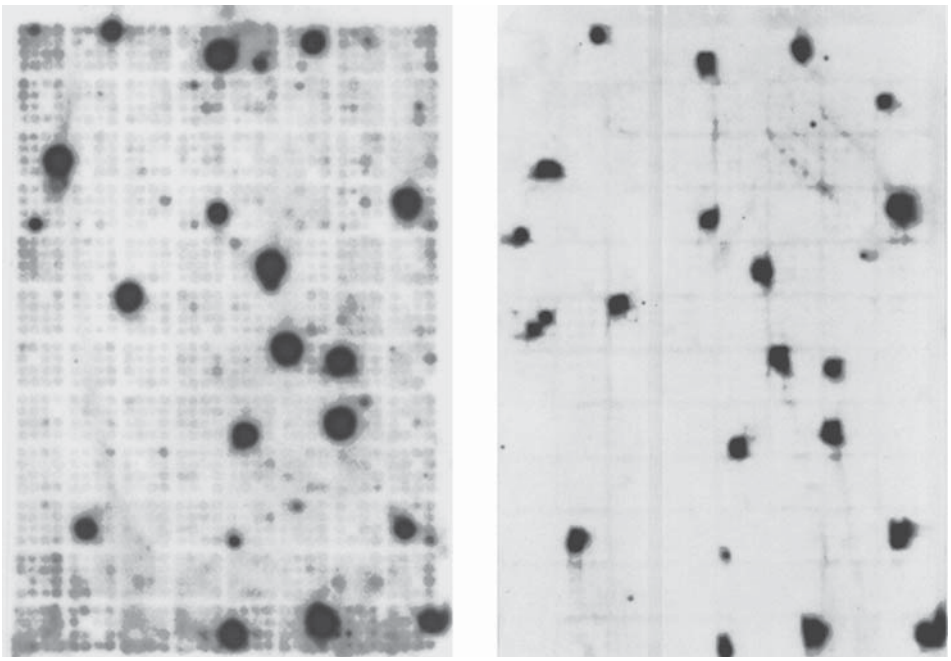


Figure 20. Comparison of High Density Colony Screens with Radioactive and Nonradioactive Probes. Almost 2000 bacterial colonies were positioned on a membrane at high density in a grid pattern. The colony pattern was screened for the presence of an interesting cloned gene with either a ^{32}P-labeled probe (panel A) or a DIG-labeled probe (panel B). (Data courtesy of Dr. Bouchier, Genethon, France.)

Result: There are only two differences in the two screens. First, the detection took 18 h with the radioactive probe, while the chemiluminescent detection took 15 min with the DIG-labeled probe. Second, two additional clones are visible in panel B (detected with the DIG-labeled probe) that are not seen in panel A; these clones proved to be real. Note that the exposures in both panels have been adjusted to show the gridlines of the colony pattern, which helped the investigator locate the positive clones.

6.2 Use of DIG-labeled Probes in Differential and Array Screening of cDNA

Differential and array cDNA screenings are often used in studies of differential gene expression, *e.g.* during development. Array screening is also used to detect differences in gene expression that are related to the effects of particular drugs or changes in physiological states. Both techniques can generate much data in a relatively short amount of time.

This section gives an overview of:

- How to use directly DIG-labeled poly(A)$^+$ mRNA or cDNA for differential screening of cDNA libraries (plaque/ colony lifts)
- How to use directly DIG-labeled mRNA for array screening of PCR-amplified cDNAs that have been spotted on nylon filters

> *By using directly-labeled mRNA as a probe, this procedure avoids the drawbacks of all array procedures that use cDNA probes. Namely, the use of a cDNA probe always carries the risk that not all the sequences in the mRNA source will be proportionally represented in the probe. Indeed, mRNA secondary structures may prevent some messages from being reverse transcribed into the probe at all.*

Topics in this section include:

3

6.2.1 Materials Required for Differential and Array Screening

Reagent / Equiment	Catalog Number	Description
For both procedures		
mRNA Isolation Kit	11 741 985 001	▶ For preparation of poly(A)$^+$ mRNA
Reagents for direct detection (quantification of probe)	–	▶ See Section 2.5 on page 85 of this chapter.
Yeast RNA	10 109 223 001	▶ Carrier nucleic acid for the hybridization buffer
20× SSC	11 666 681 001	▶ For wash buffers
Reagents and supplies for chemiluminescent detection	–	▶ See Section 4.1 on page 115 of this chapter.
For differential screening		
Titan One Tube RT-PCR Kit	11 939 823 001	▶ For preparation of cDNA
Nylon Membranes for Colony and Plaque Hybridization	11 699 075 001 (82 mm discs) 11 699 083 001 (132 mm discs)	▶ Microporous, hydrophilic, neutral nylon membranes; ideal for colony or plaque lifts
Reagents and supplies for plaque or colony lifts	–	▶ See Section 6.1 on page 131 of this chapter.
For array screening		
Nylon Membranes, Positively Charged	11 417 240 001	▶ Positively charged nylon membranes; ideal for chemiluminescent detection of DIG-labeled probes

6.2.2 Procedures

The procedures required for cDNA screening are summarized in the flow chart below. An estimate of the time required for each procedure is also given in the chart.

6.2.2.1 (page 143)	**Preparing the mRNA or cDNA Template**	▶ 1 – 2 h for mRNA ▶ Add 1 h for cDNA
6.2.2.2 (page 144)	**Differential Screening of cDNA on Nylon Membranes**	1.5 days
6.2.2.3 (page 145)	**Array Screening of cDNA on Nylon Membranes**	1.5 days

This icon means you can stop the protocol at the end of the procedure and store the product for a time before starting the next procedure.

6.2.2.1. Preparing the mRNA or cDNA Template

To prepare this template	Use this method
Poly(A)$^+$ mRNA	▶ Isolate from total RNA, cultured cells, or tissue with the mRNA Isolation Kit (Cat. No. 11 741 985 001)[1] *To prepare total RNA from a variety of sources, use either the High Pure RNA Isolation Kit (Cat. No. 11 828 665 001)[1] or the TriPure Isolation Reagent (Cat. No. 11 667 157 001 or 11 667 165 001)[1].*
cDNA	▶ Prepare from the purified poly(A)$^+$ mRNA by RT-PCR, using a cDNA Synthesis Kit such as the Titan One Tube RT-PCR Kit (Cat. No. 11 939 823 001)[1].

[1] For details, see the package insert for the product.

3

6.2.2.2. Differential Screening of cDNA on Nylon Membranes with DIG-labeled mRNA or cDNA

The following procedure is adapted from Ross, R., Ross, X.-L., Rüger, B., Laengin, T., and Reske-Kunz, A.B. *BioTechniques* 26: 150 - 155 (January 1999). It is designed to give an overview of the steps required. For detailed descriptions of differential screening procedures for specific systems, consult publications that deal with those systems.

Step	Action	Time
1	Do one of the following: **IF you...** → **THEN...** Prepared the cDNA colony or plaque library to be screened by standard methods → Go to Step 2 Are using cDNA library filters obtained from research repositories → Go to Step 3	
2	Transfer colonies, plaques, or DNA isolated from the clones to neutral nylon membranes, by doing one of the following: ▶ Prepare colony or plaque lifts in duplicate as described in Section 6.1 (page 131) of this chapter. *For replicate lifts from a single plate, leave the second membrane on the plate for 2 min (rather than 1) to allow efficient transfer in spite of the reduced number of colonies/ plaques on the plate.* OR ▶ Prepare dot blots in duplicate by placing a neutral nylon membrane on a sheet of Whatman 3MM paper and spotting up to 2 µl of DNA solution on the membrane. Air dry the membrane.	1.5 – 3.0 h
3	Prehybridize each cDNA-containing membrane with prewarmed DIG Easy Hyb at 42°C for 30 min, with gentle agitation.	30 min
4	For differential screening, hybridize different probes to duplicate cDNA-containing membranes. For each hybridization, do the following: ▶ Prepare Hybridization Solution (DIG Easy Hyb containing denatured probe). As probe, add either: ▶ 100 ng DIG-labeled mRNA/ml DIG Easy Hyb, or ▶ 40 ng DIG-labeled cDNA/ml DIG Easy Hyb *If screening arrayed cDNA libraries or if using RNA probe, add carrier [100 µg sonicated fish sperm DNA or 50 µg yeast RNA per ml] to the hybridization solution.* ▶ Replace the prehybridization solution with Hybridization Solution. ▶ Hybridize each probe to each membrane overnight in a water bath at the appropriate hybridization temperature. ***Example:*** *Use 37°C for a murine DNA probe, 42°C for a human DNA probe, or 50°C for an RNA probe.*	over night
5	Wash the membranes with the following stringency washes: ▶ Low stringency: 2× SSC containing 0.1% SDS; 2 × 10 min, room temperature. ▶ High stringency: Prewarmed buffer [0.5x SSC (for DNA:DNA) or 0.1× SSC (for RNA:DNA)] containing 0.1% SDS; 2 × 15 min, 68°C.	50 min
6	Perform chemiluminescent detection procedures with CDP-Star substrate, as detailed in Section 4.1 on page 115 of this chapter.	2 h
7	Analyze the chemiluminescent signal from the differential screens with X-ray film.	5 - 25 min

6.2.2.3. Array Screening of cDNA on Nylon Membranes with DIG-labeled mRNA

Use DMDC- or DEPC-treated water for all solutions a nd work under RNase-free conditions at all times.

Step	Action	Time
1	Prepare the cDNAs to be screened by RT-PCR; use standard methods.	Variable
2	Spot the amplified cDNAs in arrays on positively charged nylon membranes and fix them to the membrane. Do not touch the areas where cDNAs were spotted, even with gloved fingers. *This protocol may be used with a commercially available expression array, such as the CLONTECH Atlas Human cDNA Expression Array.*	Variable
3	▶ Place each cDNA-containing membrane in a hybridization bag or roller bottle. ▶ Prehybridize with 12 ml prewarmed prehybridization solution (freshly prepared DIG Easy Hyb containing 50 µg yeast RNA/ml) at 50°C for 30 min, with gentle agitation.	30 min
4	▶ Denature the DIG-labeled mRNA probe by placing in a boiling water bath for 5 min, then chilling on ice for 5 min. ▶ Prepare Hybridization Solution by adding heat-denatured probe (100 ng DIG-labeled mRNA/ml buffer) to 6 ml freshly prepared DIG Easy Hyb containing 50 µg yeast RNA. ▶ Prewarm Hybridization Solution to 50°C.	15 min
5	▶ Decant the prehybridization solution from the bag or bottle. ▶ Add prewarmed Hybridization Solution to the bag or bottle, being sure to thoroughly wet the membrane with the solution. ▶ Hybridize the probe to the membrane at 50°C overnight in a water bath with gentle agitation.	overnight
6	Wash the membranes with the following stringency washes: ▶ Low stringency: 2x SSC containing 0.1% SDS; 2 × 5 min, room temperature. ▶ High stringency: Prewarmed 0.1× SSC containing 0.1% SDS; 2 × 15 min, 68°C.	40 min
7	Perform chemiluminescent detection procedures with CDP-*Star* substrate, as detailed in Section 4.1 on page 115 of this chapter.	2 h
8	Analyze the chemiluminescent signal from the differential screens with X-ray film.	5 – 25 min

3

4

Other Nonradioactive Assays

1. Introduction

Roche offers a wide range of products for nonradioactive analysis. In addition to the DIG System applications described in earlier chapters of this guide, we offer kits and reagents for nonradioactive:

- Protein labeling and detection
- Telomere length and telomerase assays
- Gene expression analysis

In this chapter, we briefl y describe some of these nonradioactive assays. For detailed pro-tocols, see the package inserts for each assay.

The assays described in this chapter include:

For information on this topic	Turn to section	Starting on page
Nonradioactive Western Blot Assay ***Key Product:*** Lumi Light^{Plus} Western Blotting Kit	2	149
Nonradioactive Telomere Length Assay ***Key Product:*** *Telo TAGGG* Telomere Length Assay	3	151
Nonradioactive Gel Mobility Shift Assay ***Key Product:*** DIG Gel Shift Kit	4	153
Direct Immunodetection of DIG-labeled DNA on a Gel ***Key Product:*** PCR DIG Labeling Mix	5	155

2. Nonradioactive Western Blot Assay

Chemiluminescent detection has become the method of choice for detection of proteins on Western blots. Usually, in these assays, proteins are immunochemically localized on blots with target-specific primary antibodies and peroxidase-conjugated secondary antibodies. The antibody-protein complexes are then visualized with a sensitive chemiluminescent peroxidase substrate.

The best substrate for this procedure is the Lumi-Light^{Plus} Western Blotting Substrate. This substrate is highly sensitive (detects 1 – 5.0 pg of target protein). It has the added advantage of longer emission times (>12 h after addition of substrate to assay) than other Western blot substrates. Thus, the Lumi-Light^{Plus} substrate permits longer exposures or multiple exposures of a blot.

Lumi-Light^{Plus} Western Blotting Substrate is available as a separate reagent, or as part of a convenient kit [Lumi-Light^{Plus} Western Blotting Kit (Mouse/Rabbit)]. This kit contains reagents that simplify the chemiluminescent assay of Western blots.

For routine detection of more abundant proteins on a western blot, we also offer the Lumi-Light Western Blot Substrate. This substrate is still very sensitive (detects 10 - 50 pg of target protein), but not quite as sensitive as the Lumi-Light^{Plus} substrate. The Lumi-Light substrate is also long lasting (>3 h after addition of substrate to assay).

Products for This Assay

Product	Catalog Number	Description
Lumi-Light^{Plus} Western Blotting Kit (Mouse/Rabbit)	12 015 218 001	▶ Peroxidase-conjugated anti-rabbit IgG (secondary antibody) ▶ Peroxidase-conjugated anti-mouse IgG (secondary antibody) ▶ Membrane blocking reagent and wash buffer ▶ Lumi-Light^{Plus} Western Blotting Substrate
Lumi-Light^{Plus} Western Blotting Substrate	12 015 196 001	▶ Ultrasensitive chemiluminescent peroxidase substrate, for detection of rare proteins on a Western blot ▶ To be used with peroxidase-conjugated secondary antibodies and target-specific primary antibody ▶ Sensitivity: 1 – 5.0 pg of target protein
Lumi-Light Western Blot Substrate	12 015 200 001	▶ Chemiluminescent peroxidase substrate, for detection of more abundant proteins on a Western blot ▶ To be used with peroxidase-conjugated secondary antibodies and target-specific primary antibody ▶ Sensitivity: 10 – 50 pg of target protein

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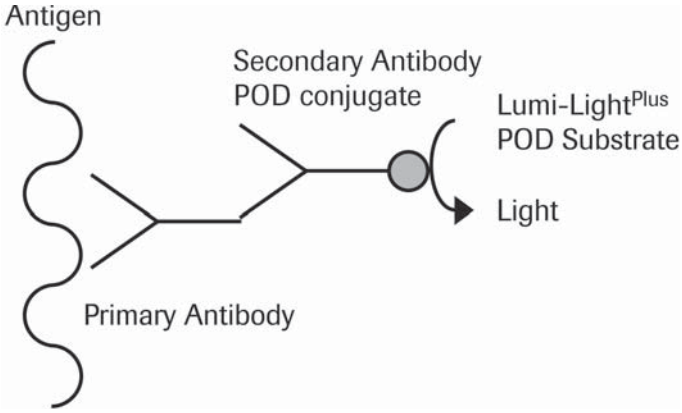
2.1 Overview of Assay

Nonradioactive detection of proteins with the Lumi-Light^{Plus} Western Blotting Kit (Mouse/Rabbit) involves the following major stages:

Step	Description[1]	Time
1	Denature samples with SDS, reducing agent, and heating to 70°C. 10 min	10 min
2	Separate denatured proteins on a gradient acrylamide-SDS gel at 200 V. *We recommend using a precast 4 – 12% NuPAGE BisTris gel (NP 0321) from Novex and the gel buffers specified by Novex.*	1 h
3	Transfer separated proteins to a PVDF membrane by electroblotting at 25 V. *We recommend using a PVDF Western Blotting membrane from Roche Apat. No. 03 010 040 001) and NuPAGE Transfer Buffer (NP 0006) from Novex.*	1 h
4	▶ Wash the blot membrane with TBS (Tris buffered saline). ▶ Block membrane with 1% membrane blocking reagent[2] to prevent nonspecific antibody binding. ▶ Wash the gel briefly with wash buffer (TBST)[2].	2 × 2 min 1 h 4 × 2 min
5	▶ Immunodetect target proteins with optimal dilution of specific primary antibody (produced in either mouse or rabbit) ▶ Use wash buffer 2 (TBST)[2] to remove unbound antibody.	1 h 4 × 2 min
6	▶ Localize primary antibody with 1:400 dilution of peroxidase-conjugated anti-mouse IgG2 or anti-rabbit IgG[2]. ▶ Use wash buffer[2] to remove unbound antibody	30 min 4 × 2 min
7	▶ Add working solution of Lumi-LightPlus Western Blotting Substrate (1:1 mix of substrate and enhancer) 2 to membrane to visualize antibody-antigen complexes.  ▶ Do one of the following to record the chemiluminescent signal: ▶ Expose membrane to X-ray film.	5 min 10 – 20 min

[1] For details of the western blotting procedure, see the package insert for the Lumi-Light^{Plus} Western Blotting Kit (Mouse/Rabbit), Cat. No. 12 015 218 001.

2 Included in Lumi-LightPlus Western Blotting Kit (Mouse/Rabbit).

3. Nonradioactive Telomere Length Assay

Telomeres are specialized DNA-protein structures found at the ends of eukaryotic chromosomes. Telomeric DNA consists of small, tandemly repeated DNA sequences (*e.g.*, human repeat sequence, TTAGGG). These G-rich sequences are highly conserved during evolution. They appear to be involved in determining the proliferative capacity and lifespan of both normal and malignant cells.

Determinations of telomere length may provide important information about normal cell aging, as well as assisting investigation of disease processes. For example, accelerated decreases in telomere length may be an indication of immune dysfunction and its associated pathologies.

We offer a *TeloTAGGG* nonradioactive assay kit for determination of telomere length in life science research applications.

We also offer two TeloTAGGG nonradioactive assays for analyzing the activity of telomerase, a ribonucleoprotein that adds telomeric repeat sequences to chromosome ends. Such assays may be useful, for example, in studying the development of malignant tumor cells from normal somatic cells in research applications.

Products for This Assay

Product	Catalog Number	Reagents included
TeloTAGGG Telomere Length Assay	12 209 136 001	▶ Two restriction enzymes which will not cut telomeric regions ▶ Restriction digestion buffer ▶ Reagents for gel electrophoresis ▶ DIG-labeled hybridization probe, specific for telomeric sequences ▶ Reagents for hybridization of probe to Southern blots ▶ Reagents for chemiluminescent ▶ detection of probe-target hybrids
DNA Isolation Kit for Cells and Tissue[1]	11 814 770 001	
Nylon Membranes, Positively Charged[1]	11 209 299 001	

[1] For more information on these products, see Chapter 3 or the Ordering Information in Chapter 2.

Other *Telo*TAGGG Kits Available

Product	Catalog Number	Description
TeloTAGGG Telomerase PCR ELISA	11 854 666 910	Assay kit for nonradioactive detection of telomerase activity
TeloTAGGG Telomerase PCR ELISA[Plus]	12 013 789 001	Assay kit for nonradioactive detection and quantification of telomerase activity

4

3.1 Overview of Assay

The nonradioactive determination of telomere length in cultured cells or tissue samples with the *Telo*TAGGG Telomere Length Assay involves the following major stages:

Step	Description[1]	Time
1	▶ Isolate genomic DNA from cell culture or tissue with the DNA Isolation Kit for Cells and Tissue.	Approx. 2.5 h
2	▶ Centrifuge the isolated DNA solution briefly in a tabletop centrifuge.	5 min
	▶ Digest isolated genomic DNA with two frequently cutting restriction enzymes[2] that will **not** cut within telomeric repeats.	2 h
3	▶ Separate restriction digest products (low molecular weight fragments plus undigested telomeric repeats) on an agarose gel.	2 – 4 h
4	▶ Depurinate the DNA in the gel.	5 – 10 min
	▶ Denature the DNA in the gel.	2 × 15 min
	▶ Neutralize the gel.	2 × 15 min
	▶ Transfer separated DNA fragments to a positively charged nylon membrane by Southern blotting (capillary transfer method).	6 – 16 h
	▶ Fix DNA to membrane by UV crosslinking or baking.	10 – 20 min
5	▶ Prehybridize blot with DIG Easy Hyb[2].	30 – 60 min
	▶ Hybridize blot with DIG-labeled probe[2] that recognizes terminal restriction fragments (TRFs).	3 h
6	▶ Detect probe-TRF complexes with alkaline phosphatase-conjugated anti-DIG antibody[2] and CDP-Star chemiluminescent alkaline phosphatase substrate[2].	Approx. 2.5 h
	▶ Record chemiluminescent signals with X-ray film.	

[1] For details of the telomerase length assay procedure, see the package insert for the *Telo*TAGGG Telomere Length Assay, Cat. No. 12 209 136 001.

[2] Included in *Telo*TAGGG Telomere Length Assay.

4. Nonradioactive Gel Mobility Shift Assay

The "gel mobility shift" or "electrophoretic mobility shift (EMSA)" assay is a rapid and simple technique for characterizing DNA-protein interactions. It is based on the differential mobility of free DNA and DNA-protein complexes in native (non-denaturing) polyacrylamide or agarose gels.

Products for This Assay

Product	Catalog Number	Reagents included
DIG Gel Shift Kit, 2nd generation	03 353 591 910	▶ Reagents for end labeling of protein-binding oligonucleotides with DIG-ddUTP ▶ Competitor DNAs, to prevent binding artifacts during gel shift assay ▶ Gel loading buffers ▶ Anti-DIG antibody, alkaline phosphatase conjugated ▶ Chemiluminescent detection reagents, including CSPD chemiluminescent alkaline phosphatase substrate ▶ Control factor Oct2A ▶ Control oligonucleotides, both DIG-labeled and unlabeled
Nylon Membranes, positively charged[1]	11 209 299 001	
DIG Wash and Block Buffer Set[1]	11 585 762 001	

[1] For more information on these products, see Chapter 3 or the Ordering Information in Chapter 2.

4.1 Overview of Assay

Step	Description	Time
1	▶ Label the 3' end of a double-stranded oligonucleotide probe or DNA fragment (ideal size, 30–100 bases) with terminal transferase and DIG-ddUTP[1]. ▶ Quantify the DIG-labeled probe[2].	15 min Approx. 2 h
2	Incubate DIG-labeled oligonucleotide (containing the protein binding sequence) with a cell extract or partially purified DNA binding protein (*e.g.* control factor Oct2A)[1]. *Adding competitor DNA [poly d(A-T) or poly d(I-C)] prevents formation of nonspecific DNA-protein complexes, especially in cell extract samples.*	15 min
3	Separate the assay mixture on a native (non-denaturing) polyacrylamide gel at 70 V in 0.5x TBE buffer. *We recommend using 5x High-Density TBE Sample Buffer (LC6678), precast 6% DNA Retardation Gel (EC6365), and XCell II Mini Cell (E19001) from Novex. TBE running buffer is available at 10x concentration (Cat. No. 11 666 703 001) from Roche.*	50–60 min
4	Transfer separated components to a positively charged nylon membrane by electroblotting at 30 V in 0.5x TBE buffer. *We recommend using the XCell II Blot Module (E19051) from Novex.*	1 h
5	Detect2 DIG-labeled DNA-protein complexes with alkaline phosphatase-conjugated anti-DIG antibody and CSPD chemiluminescent alkaline phosphatase substrate. *The detection procedure also uses components of the DIG Wash and Block Buffer Set.*	Approx. 2.5 h
6	Record the chemiluminescent signals with X-ray film.	5–20 min

[1] For details of the nonradioactive gel mobility shift assay procedure, see the package insert for the DIG Gel Shift Kit, Cat. No. 11 635 352 001. Reagents are included in DIG Gel Shift Kit.

[2] For details of this procedure, see Section 4.1 of Chapter 3.

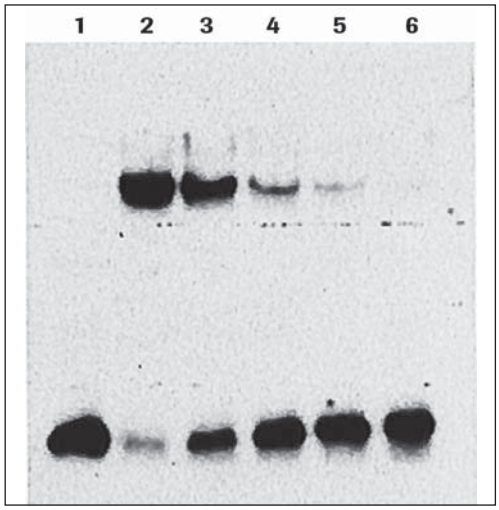


Figure 21. DIG Gel Shift Assay with Oct2A Control Protein and Oligonucleotide. Gel mobility shift assays were performed with the control Oct2A protein and varying amounts of Oct2A-binding oligonucleotide (included in the DIG Gel Shift Kit). The reaction components were separated on a 12.5% native polyacrylamide gel, then transferred to a nylon membrane by electroblot. DIG-labeled moieties were detected in a chemiluminescent assay (CSPD substrate, 30 min exposure, X-ray film). Lane 1: DIG-labeled control oligonucleotide (0.8 ng) containing Oct2A binding site. Lane 2: DIG-labeled control oligonucleotide (30 fmol) after incubation with 50 ng Oct2A control protein. Lanes 3–6: Same as lane 2, except the incubations also contained increasing amounts of unlabeled control nucleotide (25-, 62-, 125-, and 250-fold excess, left to right). Upper band, protein-oligonucleotide complex; lower band, unbound oligonucleotide.

Result: The Oct2A control protein specifically bound a site on the DIG-labeled control oligonucleotide (lane 2). The reaction was specific because the protein could be competed off the labeled oligonucleotide by increasing amounts of unlabeled oligonucleotide (lanes 3–6).

5. Direct Detection of a DIG-labeled DNA

DIG labeling of PCR products and direct immunodetection of the DIG-labeled DNA transferred to nylon membrane offer a thousandfold increase in sensitivity over detection by ethidium bromide staining. (See Section 6.2, "Typical Results with the PCR DIG Labeling Mix" below.)

Products for This Assay

Product	Catalog Number	Reagents included
PCR DIG Labeling Mix, 10× conc.	11 585 550 910	Mixture (10× conc.) of nucleotides in water, pH 7, including: ▶ dATP, 2 mM ▶ dCTP, 2 mM ▶ dGTP, 2 mM ▶ dTTP, 1.9 mM ▶ DIG-11-dUTP, alkali-stable, 0.1 mM
Anti-Digoxigenin-AP, Fab fragments[1]	11 093 274 910	
Nylon Membrane, positively charged[1]	11 209 299 001	
Ready-to-use CDP-*Star*[1]	12 041 677 001	
DIG Wash and Block Buffer Set[1]	11 585 762 001	

[1] For more information on these products, see Chapter 3 or the Ordering Information in Chapter 2.

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5.1 Overview of Assay

Direct immunodetection of DNA prepared with the PCR DIG Labeling Mix involves the following major stages:

Step	Description	Time
1	Amplify a target DNA by PCR with a reaction mix containing the PCR DIG Labeling Mix[1].	Approx. 2 h
2	Separate the samples on an agarose gel (in TBE or TAE buffer).	30 min –2 h
3	Transfer separated components to a positively charged nylon membrane by capillary transfer2. *You do not need to denature the DNA before transfer.*	overnight
4	Detect[2] DIG-labeled DNA-protein complexes with alkaline phosphatase-conjugated anti-DIG antibody and CDP-Star chemiluminescent alkaline phosphatase substrate. *The detection procedure also uses components of the DIG Wash and Block Buffer Set.*	Approx. 2.5 h
5	Record the chemiluminescent signals with X-ray film	5–20 min

[1] For details of the labeling procedure, see the package insert for the PCR DIG Labeling Mix, Cat. No. 11 585 550 001.

[2] For details of these procedures, see Sections 3.1 and 4.1 of Chapter 3.

Comparison of Sensitivity, Direct Detection of DIG-labeled Products vs. Ethidium Bromide Staining

Detection Method	Could Detect This PCR Product	Produced from
Ethidium bromide staining	Unlabeled DNA on a gel	1 pg template
Direct immunodetection	DIG-labeled DNA on a blot	1 fg template

5.2 Relative Sensitivity of Hybridization Probes Prepared with the PCR DIG Probe Synthesis Kit and the PCR DIG Labeling Mix

Use the PCR DIG Labeling Mix **only** to label PCR products that will be directly detected. Do **not** use it to label PCR products that will be used as hybridization probes.

Explanation: The ratio of DIG label to unlabeled nucleotide (DIG-dUTP:dTTP) in the PCR DIG Labeling Mix is very low (1:20). This allows low level DIG labeling without significantly altering the molecular weight of the labeled product. This level of DIG labeling is sufficient for direct detection, but is inadequate (Figure 22) for producing the sensitive hybridization probes needed to detect single-copy genes on Southern blots (or rare RNAs on Northern blots).

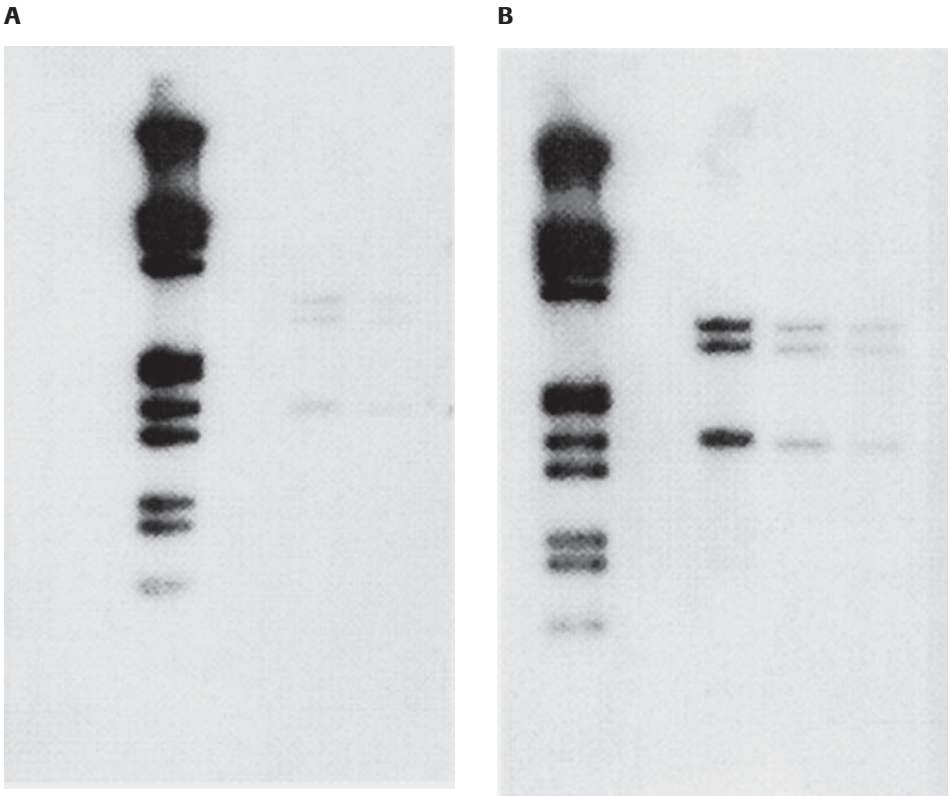

Figure 22. Comparison of the Sensitivity of DIG-labeled Probes Generated with Different Concentrations of DIG-dUTP. We compared the sensitivity of two probes, one prepared with the 1:3 DIG-dUTP:dTTP ratio found in the PCR DIG Probe Synthesis Kit (Panel B), and the other with the 1:20 DIG-dUTP:dTTP ratio found in the PCR DIG Labeling mix (Panel A). Each probe was 442 bp and recognized a human tPA sequence. The probes were used to detect the target sequence in different amounts of human DNA (10, 5, and 2.5 µg, left to right in each panel) on a Southern blot. A DIG-labeled DNA Molecular Weight Marker was included at the left of each blot.

Result: The probe prepared with 1:3 DIG-dUTP:dTTP could easily detect the three tPA bands in 2.5 µg DNA (panel B). The probe prepared with 1:20 DIG-dUTP:dTTP could only detect the tPA fragments as faint bands in the 10 and 5 µg samples; it could not detect the fragments in the 2.5 µg sample at all.

Conclusion: The PCR Labeling Mix does not produce probes that are sensitive enough to detect single-copy genes in complex samples. **For labeling of high sensitivity hybridization probes, always use the PCR DIG Probe Synthesis Kit (as described in Section 2.2, Chapter 3).**

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Appendix

A: Summary of DIG Basics

The tables below summarize the information given in Chapter 2 of this guide. For a more complete discussion of DIG basics, see Chapter 2.

1. DIG Labeling

DIG Labeling Methods: Template Requirements and Probe Sensitivity

Method	Amount of Template Required	Labeled Probe Can Detect
PCR Labeling	▶ Plasmid DNA: **10 pg** (range, 10–100 pg)[1] ▶ Genomic DNA: **10 ng** (range, 1–50 ng)[1]	0.1 – 0.03 pg DNA
Random Primed Labeling (DIG-High Prime)	▶ 10 ng – 3 µg DNA	0.1 – 0.03 pg DNA
RNA Labeling	▶ Linearized plasmid DNA: 1 µg ▶ PCR product with promoter: 100 – 200 ng	0.1 – 0.03 pg DNA 0.1 – 0.03 pg RNA
Nick Translation	▶ 0.1 – 2 µg DNA	Mainly used for in situ applications; sensitivity depends on target system
3' End Labeling	▶ 100 pmol oligonucleotide	10 pg DNA or RNA
3' Tailing	▶ 100 pmol oligonucleotide	1 pg DNA or RNA

[1] Figure in **bold type** is preferred amount. For best results, keep target amounts low.

2. Electrophoresis and Blot Transfer

Amount of Target Nucleic Acid To Load on Gel

Type of Blot	Type of DIG-labeled Probe	Amount of Target To Load	Comment
Southern	DNA or RNA	▶ Plasmid DNA: <1 ng ▶ Genomic DNA: 1.0, 2.5, 5.0 µg (3 separate samples) ⚠ *Never load more than 10 µg genomic DNA*	A 10 µg sample of human genomic DNA contains 10 pg of a single-copy, 3 kb gene. Since DIG-labeled probes detect 0.1 pg target, a 3 kb target could easily be detected in a 10 µg (or a 1 µg) sample.
Northern	RNA	▶ Total RNA: 1 µg ▶ mRNA: 100 ng ⚠ *Never load the amounts of total RNA (10 – 20 µg) or mRNA (1 µg or more) you would use with a radioactive probe.*	A DIG-labeled RNA probe can detect: ▶ An abundant mRNA (actin mRNA) in as little as 5 ng total RNA ▶ A rare mRNA (CTF1 mRNA) in as little as 50 ng total RNA
	DNA	▶ Total RNA: 5 µg ▶ mRNA: 500 ng ⚠ *Never load the amounts of total RNA or mRNA you would use with a radioactive probe.*	

3. Hybridization of Probe to Target

Concentration of DIG-labeled Probe during Hybridization

Probe Type	Probe Concentration
Random primed labeled DNA	25 ng/ml
PCR-labeled DNA	2 µl /ml
Transcriptionally labeled RNA	20 – 100 ng/ml
End labeled oligonucleotides	1 – 10 pmol/ml
Tailed oligonucleotides[1]	0.1 – 10 pmol/ml

[1] For tailed oligonucleotide, add 0.1 mg/ml poly(A) and 5 µg/ml poly(dA) to prehybridization buffer and hybridization buffer. Polynucleotides prevent nonspecific signals that can be generated by the tails.

Typical Hybridization Time

Application	Recommended Hybridization Time[1,2]
DNA:DNA hybridizations	
Single copy gene detection	ON (12 – 16 h)
DNA fingerprinting (multiple locus probes)	2 – 4 h
DNA fingerprinting (single locus probes)	ON (12 – 16 h)[3]
Colony/Plaque hybridization	At least 4 – 6 h (up to ON)
RNA:RNA hybridization	At least 4 – 6 h (up to ON)
Oligo:DNA hybridization	At least 1 – 6 h (up to ON)

[1] Prehybridization time for all applications (without probe) = 30 min.

[2] ON = overnight.

[3] Complete in 4–6 h with DIG Easy Hyb.

Optimal Hybridization Temperature[1]

Type of Hybrid	Optimal Temperature
DNA:DNA	42°C (range, 37° – 42°C)
DNA:RNA	50°C
RNA:RNA	68°C

[1] These temperatures are correct for hybridization to mammalian nucleic acid with a 40% GC content in the presence of DIG Easy Hyb or Standard Buffer containing 50% formamide. For calculation of other hybridization temperatures, see below.

Calculation of Optimal Hybridization Temperature for DNA:DNA Hybrids

The optimum hybridization temperature (T_{hyb}) for a probe-target hybrid can be calculated from the formulas below. Please note that the formula you use depends on the hybridization buffer you use:

If you are using this hybridization buffer	Always use this calculation for T_{hyb}
DIG Easy Hyb	▶ Calculation 1
Any buffer except DIG Easy Hyb	▶ Calculation 2

Calculation 1 (for Easy Hyb only)

Always use these equations to calculate the optimal hybridization temperature (T_{hyb}) for DNA:DNA hybrids **in DIG Easy Hyb**.

$T_m = 49.82 + 0.41\ (\%\ G + C) - 600/l$

$T_{hyb} = T_m - (20°\ \text{to}\ 25°C)$

Where:
T_m = melting point of probe-target hybrid

(% G + C) = % of G and C residues in probe sequence

T_{hyb} = Optimal temperature for hybridization of probe to target in DIG Easy Hyb

This formula holds for probes with a 40% GC content and 80 – 100% homology to target. For sequences that are <80% homologous, the T_{hyb} will be lower than that calculated above (approx. 1.4°C lower per 1% mismatch) and must be determined empirically.

l = length of hybrid in base pairs

Example 1: Assume hybridization buffer is DIG Easy Hyb, % G + C (probe) = 50%, and L = 72 bp. Then:

$T_m = 49.82 + 0.41\ (50) - 600/72 = 62°C$ (in DIG Easy Hyb)

$T_{hyb} = 62° - (20°\ \text{to}\ 25°) = 42°\ \text{to}\ 37°C$ (in DIG Easy Hyb)

Calculation 2 (for any buffer except DIG Easy Hyb)

Use these equations to calculate the optimal hybridization temperature (T_{hyb}) for DNA:DNA hybrids **in any buffer except DIG Easy Hyb**.

$T_m = 16.6 \log [\text{Mol Na+}] + 0.41 (\% \text{ G + C}) + 81.5$

$T_{hyb} = T_m - 25°C$

Where:
T_m = melting point of probe-target hybrid (in the absence of formamide)

The addition of 1% formamide will lower the calculated T_m by 0.72°C. The addition of 50% formamide will lower the calculated T_m by 36°C (0.72°C × 50).

Mol Na^+ = molar concentration of sodium in hybridization buffer

(% G + C) = % of G and C residues in probe sequence

T_{hyb} = Optimal temperature for hybridization of probe to target

This formula holds for probes that are approximately 80–100% homologous to target; i.e. the formula allows up to an 20% mismatch between the sequences. For sequences that are < 80% homologous, the T_{hyb} will be lower than that calculated above (approx. 1.4°C lower per 1% mismatch) and must be determined empirically.

Example 2: Assume hybridization buffer is 3× SSC (Mol Na^+ = 0.585 M) and % G + C (probe) = 50%. Then:

$T_m = 16.6 \log [0.585] + 0.41 (50) + 81.5 = 98°C$

$T_{hyb} = 98° - 25° = 73°C$ (in the absence of formamide)

$T_{hyb} = [98° - 0.72° (50)] - 25° = 37°C$ (in the presence of 50% formamide)

Calculation of Optimal Hybridization Temperature for Oligonucleotide Probes

$T_m = 4°C (\#GC) + 2°C (\#AT)$ [in DIG Easy Hyb]

$T_{hyb} = T_m - 10°C$ [in DIG Easy Hyb]

Where:
#GC = Number of G and C residues in oligo probe

#AT = Number of A and T residues in oligo probes

4. Stringent Washes of Blot

Low Stringency Washes

Type of Hybrid	Buffer	Duration	Temperature
All	2× SSC containing 0.1% SDS	2 × 5 min	Room temperature

High Stringency Washes[1,2]

Type of Hybrid	Buffer	Duration	Temperature
DNA:DNA	0.5 SSC + 0.1% SDS	2 × 15 min	65°C
RNA:DNA or RNA:RNA	0.1× SSC + 0.1% SDS	2 × 15 min	68°C
Oligo:DNA	At least 1× SSC[3] + 0.1% SDS	2 × 15 min	Temperature used for hybridization *Optimal temperature can vary.*

[1] For mammalian DNA that is 100% homologous to probe.

[2] Always preheat washes before adding to membrane.

[3] In most cases, you may use the more stringent 0.5× SSC.

5. Sensitivity of Alkaline Phosphatase Substrates

Comparative Sensitivity of Alkaline Phosphatase Substrates in DIG System[1]

Substrate	Can Detect	Duration of Assay
NBT/BCIP (chromogenic)	▶ 0.1 pg DNA on a dot blot	16 h
	▶ Single-copy gene in 1 µg human DNA on a Southern blot	
	▶ Colonies/plaques in a screen of a library with low number of vector copies/cell	
CSPD (chemiluminescent)	▶ 0.03 pg homologous DNA in 50 ng heterologous DNA on a Southern or dot blot	< 30 min[2]
	▶ Single-copy gene in 0.3 µg human DNA on a Southern blot	
	▶ 0.1 pg RNA on a Northern blot	
CDP-*Star* (chemiluminescent)	▶ 0.03 pg homologous DNA in 50 ng heterologous DNA on a Southern or dot blot	< 5 min[2]
	▶ Rare mRNA in 100 ng total RNA on a Northern blot (RNA probe)	15 min[2]

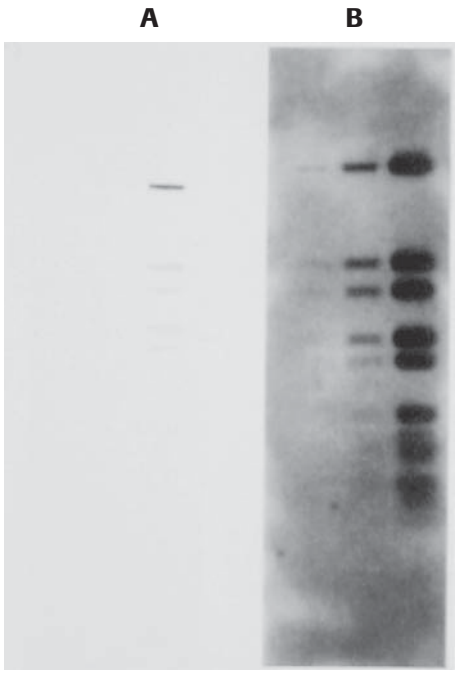
[1] Data gathered under optimized hybridization conditions.

[2] X-ray film exposure.

B: Troubleshooting the DIG System

1. Possible Problems and Recommendations

Problem 1: Too weak or too strong signals



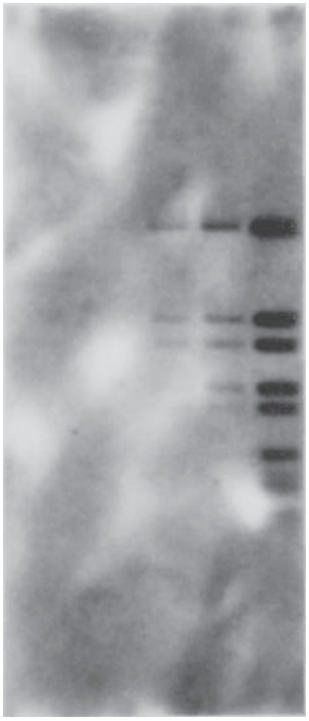
Possible cause

Chemiluminescent assay exposure time too short (A) or too long (B).

Recommendation

Increase [for (A)] or decrease [for (B)] the amount of time you expose the blot to X-ray film.

Problem 2: Uniform High Background



Possible causes

- Probe concentration was too high during hybridization (most likely cause).
- Template DNA was contaminated, leading to impure probe.

Recommendation

- Reduce probe concentration
- Perform a mock hybridization with different concentrations of probe (as described in Part 2 of chapter 5 B, page 177) to determine the amount of probe that gives the most signal with the least background.
- Never use the entire yield from a labeling reaction to analyze a single blot (as you might for radioactive analysis)
- Purify the probe with proteinase K treatment and/or filtration through a 0.45 µm cellulose acetate filter or use the High Pure PCR Product Purification Kit.
- Purify the template by phenol extraction and ethanol precipitation.

Problem 3: Irregular, Cloudy Background

Possible cause

Uneven distribution of probe during hybridization, caused by not using enough hybridization solution or by letting the membrane dry during incubation.

Recommendation

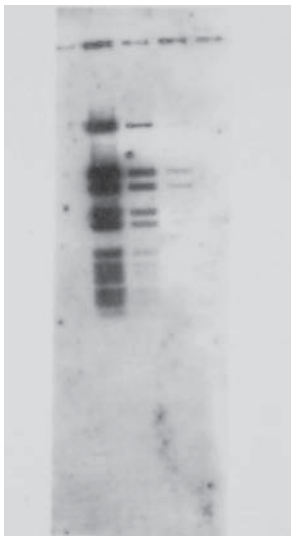
- Do not add probe directly to prehybridization solution.
- Do not allow membrane to dry between prehybridization and hybridization. For instance, do not pour off the prehybridization solution until the hybridization solution is ready for immediate addition to the membrane.
- Use at least 3.5 ml of hybridization solution per 100 cm² of membrane.

If using roller bottles for incubation, use at least 6 ml hybridization solution per bottle.

- Shake the hybridization container during the hybridization incubation.

Make sure that the hybridization bag lies flat in the bottom of the water bath.

Problem 4: Irregular, Smeared, Grainy Background



Possible causes

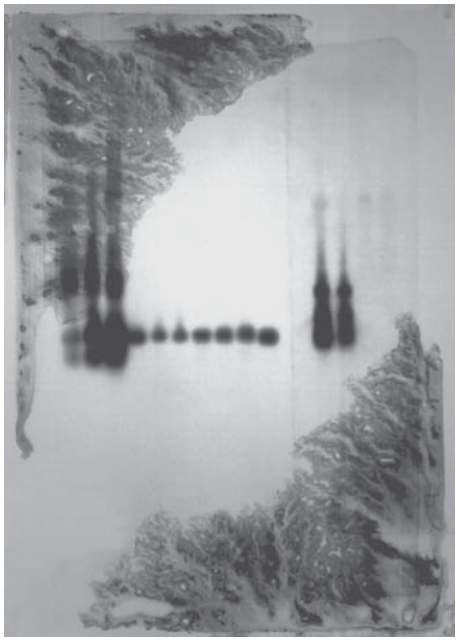
- Non-uniform distribution of chemiluminescent substrate, *e.g.* due to performing incubation while membrane wrapped in plastic wrap.
- Wrinkles in the hybridization bag, causing uneven contact between membrane and X-ray film.
- Drying of membrane during chemiluminescent visualization procedure.

The grainy appearance of the background indicates that the drying occurred during the chemiluminescent procedure rather than during hybridization. Drying during hybridization leads to a cloudy background (as shown in Problem 3).

Recommendation

- Do not wrap membrane in plastic wrap during incubation with chemiluminescent substrate.
- Spread the chemiluminescent substrate uniformly over the surface of the membrane, as described in Section 4.1, page 115 of Chapter 3.
- Before exposing bag to X-ray film, flatten any wrinkles between blot and membrane by rolling a pipette over the surface of the bag.
- Carefully seal the damp membrane (in a folder or bag) during the incubation/exposure to X-ray film. Check the seals to make sure liquid cannot leak.

Problem 5: High Background on Only Part of Membrane



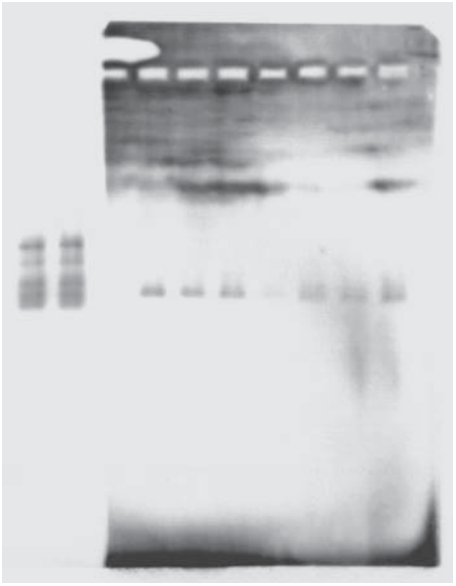
Possible cause 1

(Data courtesy of Dr. Bacchetti and Dr. Marusic, McMaster University, Canada.) Drying of membrane during chemiluminescent detection procedure.

Recommendation

- Do not wrap membrane in plastic wrap during incubation with chemiluminescent substrate. Plastic wrap cannot be sealed and will allow membrane to dry out.
- Carefully seal the damp membrane (in a development folder or hybridization bag) during the incubation/exposure to X-ray film. Check the seals to make sure liquid cannot leak.

[Problem 5 (cont.)]



Possible cause 2

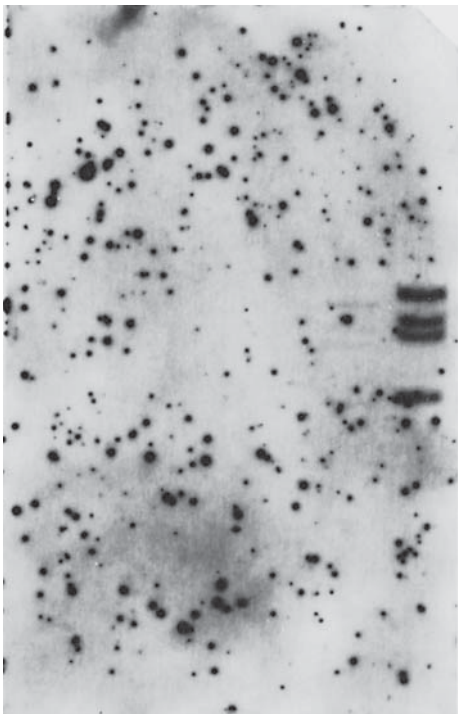
(Data courtesy of Dr. Bacchetti and Dr. Marusic, McMaster University, Canada.)

Membrane dried while in blocking solution and stuck to side of incubation tray.

Recommendation

- Never let membrane dry at any stage of the prehybridization, hybridization, or detection procedures.
- Always use enough liquid in each incubation to cover membrane completely.
- Control the membrane occasionally during incubations (especially those with agitation) to ensure it does not dry or stick to the incubation tray.

Problem 6: Spotty Background



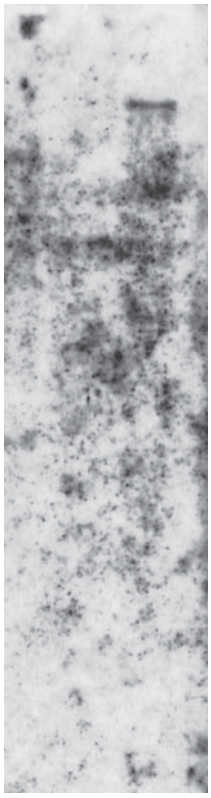
Possible cause 1

Antibody contained a precipitate when it was applied to the membrane.

Recommendation

- Before each use, centrifuge the antibody preparation (in its original vial) for at least 5 minutes at 10 000 rpm. Take an aliquot from the surface of the supernatant for the antibody dilution.
- Be sure the Detection Buffer used after the antibody incubation step does not contain Mg^{2+} ions.

[Problem 6 (cont.)]



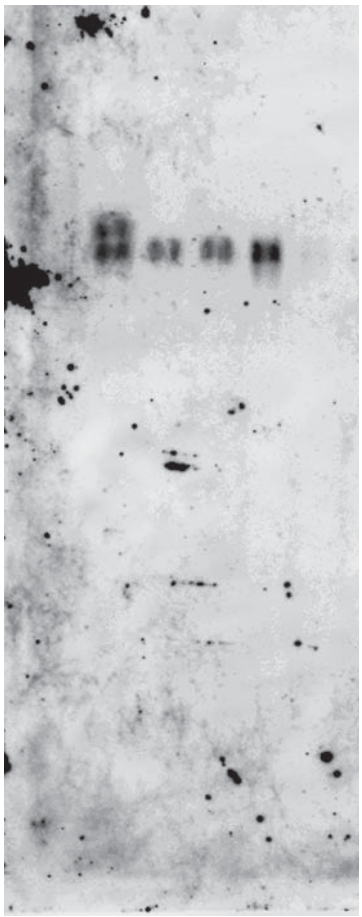
Possible cause 2

Membrane was unsuitable for nonradioactive assay.

Recommendation

Use positively charged Nylon Membranes from Roche.

[Problem 6 (cont.)]



Possible cause 3

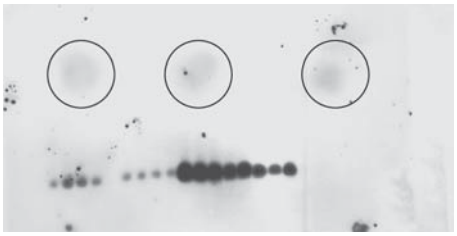
(Data courtesy of T. Ruckes, Institute of Virology, Erlangen, Germany.)

Salt crystals (from 20× SSC) were baked into membrane before the detection procedure.

Recommendation

- Wash the membrane briefly in 2× SSC before baking.
- Do not bake the membrane. Instead, fix the DNA by UV crosslinking, then rinse the membrane with water (as described in Section 3.1.2.2, page 97 of Chapter 3).

Problem 7: Gray Circles above Bands



(Data courtesy of Dr. Bacchetti and Dr. Marusic, McMaster University, Canada.)

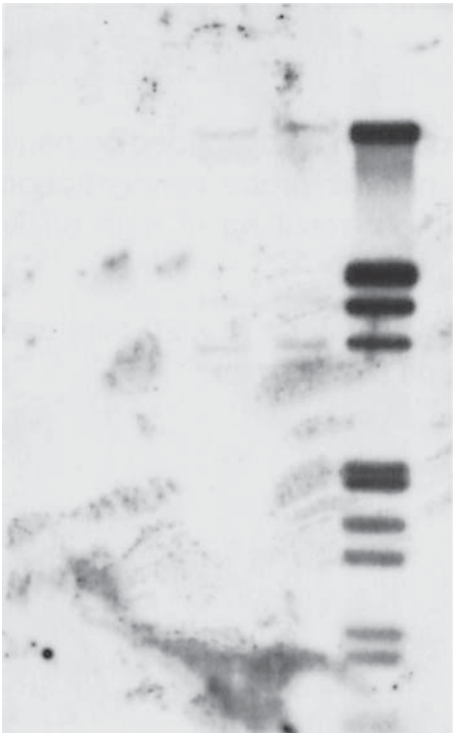
Possible cause

Membrane was too dry before chemiluminescent substrate was added. The substrate dried at several spots on the membrane, leading to the gray circles.

Recommendation

- Do not let the membrane dry (even slightly) before adding the chemiluminescent substrate.
- Cover the membrane with the second sheet of the folder or bag **immediately** after you add the chemiluminescent substrate (as described in Section 4.1, page 115 of Chapter 3). Even a little dried substrate can lead to gray circles (especially if you are using CDP-*Star*).

Problem 8: Spots on the X-ray Film, in Areas Not Covered by Membrane



Possible cause

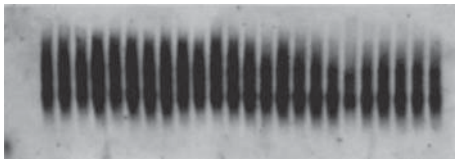
Electrostatic charge on the outside of the sealed hybridization bag.

Recommendation

- Wipe the surface of the sealed bag with 70% ethanol before incubating it with the X-ray film.
- When handling the membrane, always wear gloves.
- Use forceps, never fingers, to grip the membrane.
- Grip only the edges of the membrane, never the center (even with the forceps).

Problem 9: No Background on Membrane, but Strong, Nonspecific Smear across Lanes

(Data courtesy of Dr. B. Hengerer, Ciba Geigy, Switzerland.)



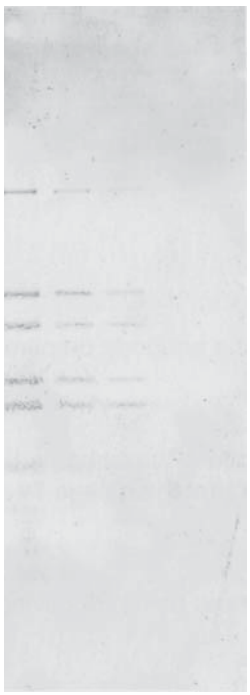
Possible causes

- Probe binds to some other nucleic acid in sample besides the (desired) target.
- Too much target nucleic acid on gel.
- Target nucleic acid partially degraded.

Recommendation

- Reduce the amount of target loaded on gel.
- Reisolate target nucleic acid and check the sample for degradation before using it in the hybridization experiment.

Problem 10: Diffuse Bands



Possible causes

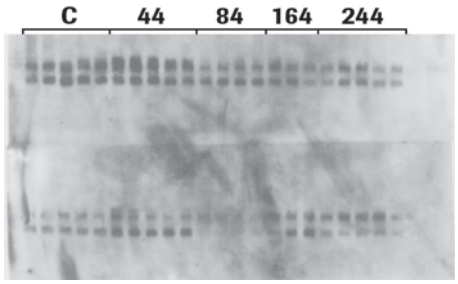
- Incomplete transfer of nucleic acid during blot transfer.
- Mashing of gel during blot transfer.
- Partial drying of gel during vacuum transfer.

Recommendation

- During a capillary transfer, make sure the weight is evenly distributed over the surface of the blot and does not mash or otherwise distort the gel.
- During a vacuum transfer, be sure the vacuum is constant. Also, be sure the vacuum is strong enough to effect the transfer before the gel dries unevenly.

Problem 11: Missing Bands

(Data courtesy of Dr. B. Hengerer, Ciba Geigy, Switzerland.)



Possible causes

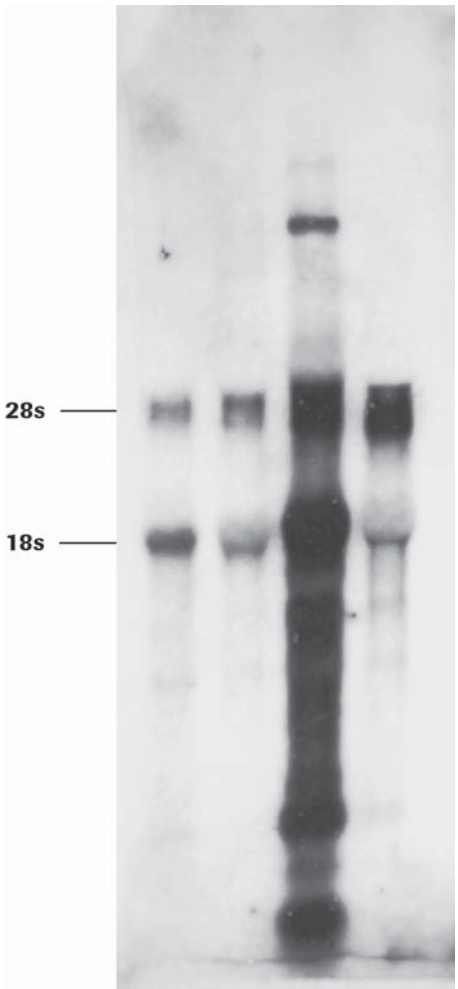
- Air bubble was trapped between the membrane and gel during blot transfer.
- Depurination with HCl not performed (if missing bands are > 5 kb).

Recommendation

- Always set up the blot transfer "sandwich" carefully and eliminate all air bubbles trapped between the surface of the gel and membrane.
- If DNA on gel is large (> 5 kb), treat the gel with 0.25 M HCl, 10 – 20 min, before starting blot transfer. This depurinates the DNA, so it will be fragmented during the subsequent alkaline denaturation step and will transfer easily to the blot (see Section 3.1.2.2, page 97 of Chapter 3, for details).

Problem 12: Unwanted rRNA Hybridization Signals on a Northern Blot

(Data courtesy of M. Block, University of Hamburg, Germany.)



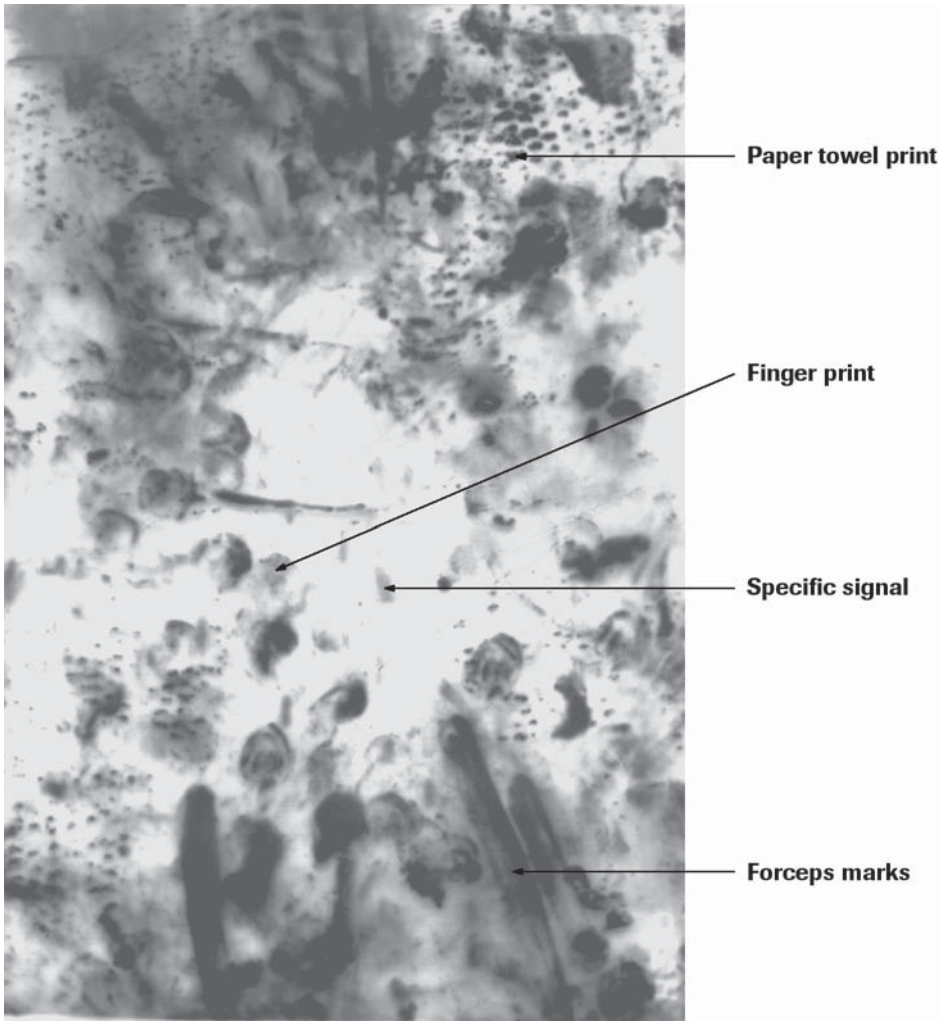
Possible cause

Labeled probe contained unwanted RNA or chromosomal DNA sequences (from *E. coli*), which bound ribosomal RNA in the samples.

Recommendation

- Prepare labeled probe from a plasmid DNA preparation that is free of contaminating chromosomal DNA and RNA, *e.g.* purify the plasmid with the High Pure Plasmid Isolation Kit.
- Instead of a DNA probe, use an RNA probe prepared by transcriptional labeling (as in Section 2.3, page 74 of Chapter 3).
- Do not exceed the recommended target RNA loads
- For DNA probes: 5 µg total RNA or 500 ng mRNA
  For DIG labeled RNA probes: 1µg total RNA or 100 ng mRNA

Problem 13: The Membrane Strikes Back



Possible causes

- Lack of care in handling membrane during procedure.

  Every scratch, touch, or gouge on the surface of the membrane will be made visible during the chemiluminescent detection/visualization procedure.

- Blot that had previously been hybridized only with radioactively labeled probes was rehybridized with a DIG-labeled probe.

  All these background "marks" were not visible in the radioactive detection procedure.

Recommendation

- Be very careful when handling the blot during a DIG procedure. Handle it only by the edges, and only with gloves and forceps. Do not touch the experimental portion of the blot with **anything**.
- Always start with a fresh blot when performing a DIG procedure for the first time; do not reuse a blot from a previous radioactive detection procedure. Membrane damage that is invisible during a radioactive procedure may be visible in a DIG procedure.

2. How To Optimize Single Copy Gene Detection Easily

This section illustrates how you can evaluate DIG-labeled probes to ensure good results without many optimization steps.

To illustrate the effectiveness of this optimization process, we used it on some "problem" random-primed labeled probes from a customer. The customer actually sent us four different templates. All four templates were designed to produce probes that recognize an RFLP associated with a single-copy genome. The customer complained that, in his hands, all four labeled probes produced a high background on the hybridization membrane.

We followed these steps to optimize the DIG-labeled probes:

1. We determined the labeling efficiency of the probe(s) by the direct detection method (Figure A).
2. From the result of Step 1, we chose the probe (probe 2) with the greatest sensitivity. We performed a "mock hybridization" with different concentrations of probe 2 to determine which concentration gives minimal background (Figure B).
3. We also tried filtering the probe through cellulose acetate to try to reduce the observed background (Figure B).

   ⚠ *Do not use a nitrocellulose membrane to filter the probe, since the probe will stick irreversibly to nitrocellulose.*

To verify the effectiveness of our optimization procedure, we tested the optimized probe in an actual Southern blot experiment (Figure C). The blot shows good results. The optimization procedure worked. It will also work on your DIG-labeled probe.

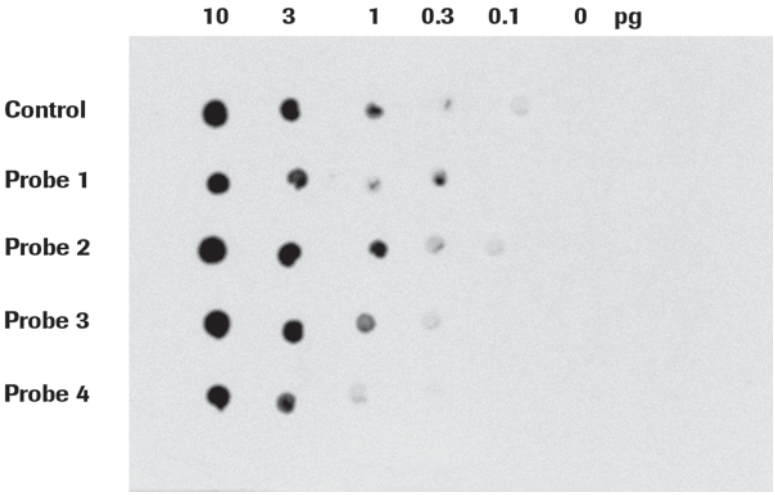


Figure A. Initial Evaluation of Four Probes for Detecting Single-locus RFLPs. DIG-labeled probes were checked for labeling efficiency in a direct detection assay (Chapter 3, page 85).

Result and conclusions: Probe 2 has the best sensitivity (0.1 pg DNA). Probes 1 and 3 have a sensitivity of 0.3 pg, which is still enough to detect single-copy genes. Probe 4 had a sensitivity of only 1 pg. Therefore, probes 1 – 3 all had enough sensitivity to be used in the detection of single-copy genes. Probe 4 did not have sufficient sensitivity for detection of single-copy genes.

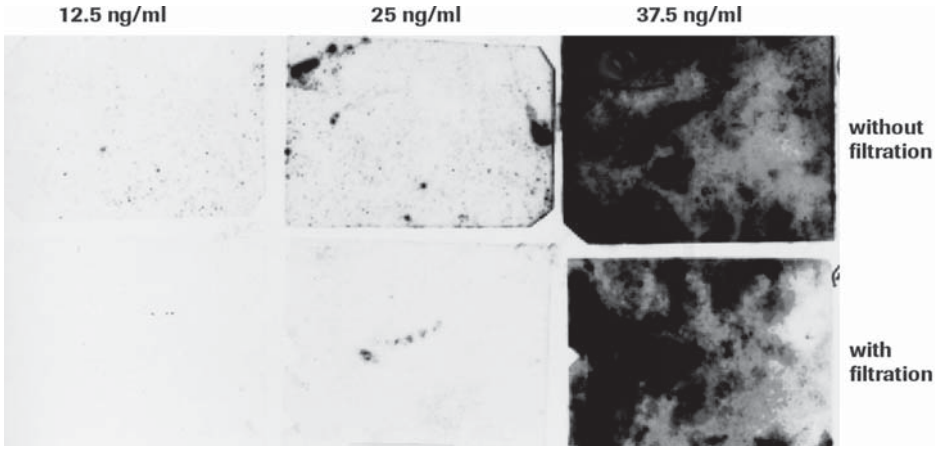


Figure B. Mock Hybridization with the Most Sensitive DIG-labeled Probe. DIG-labeled probe 2 (from Figure A) was used as probe in a series of standard hybridization reactions. However, the "blot membranes" in each test actually contained no target DNA. This serves as a test for non-specific background binding of the probe.

Several concentrations of probe in DIG Easy Hyb were used in the mock hybridization. In each case, stringent hybridization and wash conditions were used. The upper series of photos shows the results when the different probe concentrations were used without prior treatment of the probe. The lower series of photos were obtained when the same probe was first filtered through a 0.45 µm cellulose acetate filter and then used at different concentrations. The three probe concentrations used in each series were the standard concentration (25 ng/ml, middle panel), 0.5× standard concentration (12.5 ng/ml, left panel), and 1.5× standard concentration (37.5 ng/ml, right panel).

Result and conclusions: The acceptable range of concentrations for the probe is very narrow. Both the low (left panel) and standard (middle panel) probe concentration produced acceptable background. In contrast, the high concentration (right panel) produced a very high background. The difference between a good background and a bad background required a change of 50% or less in probe concentration. This background is clearly due solely to the excessive concentration of probe in the hybridization reaction.

A comparison of the upper and lower series of panels shows that filtration lowered the background even further. The unfiltered probe (upper series of photos) produces a lot of spotty background. The "pepper spot" background is caused by impurities in the template used for labeling. Some of the contamination could be removed from the labeled probes by simple cellulose acetate filtration (lower series of photos).

Therefore, probe 2 should not be used at a concentration greater than the standard concentration of 25 ng/ml and should be filtered through cellulose acetate before use.

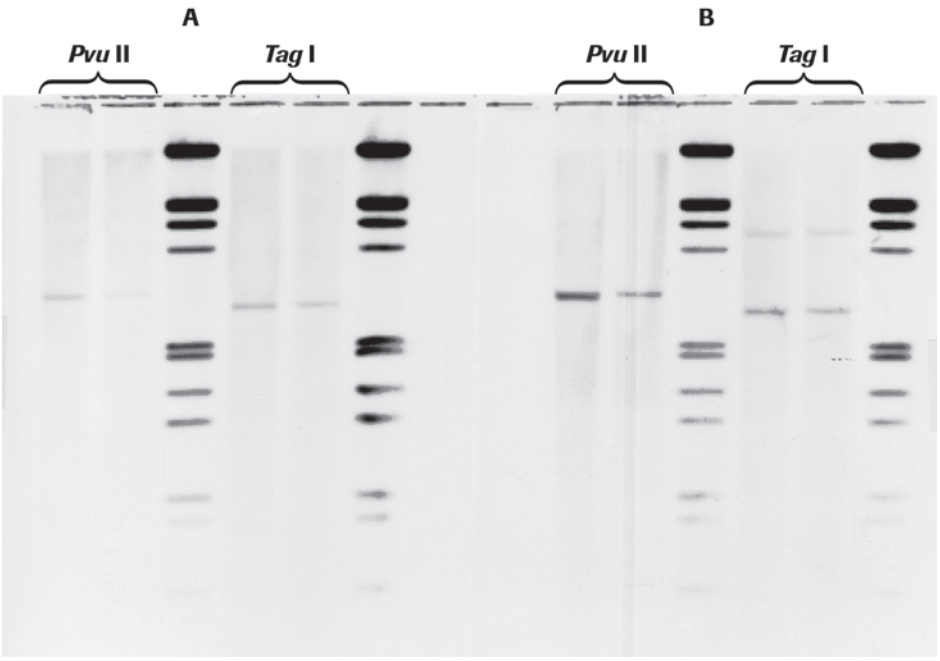


Figure C. Southern Blot Performed under Optimal Conditions with the Most Sensitive DIG-labeled Probe. Filtered, DIG-labeled probe 2 was used to detect a single-copy gene on a Southern blot. The target DNA was either DNA from human blood (panel A) or DNA from HeLa cells (panel B). In each case, samples containing either 5 or 2.5 µg of DNA were digested with the indicated restriction enzyme (*Pvu* II or Taq I), separated on a 1% agarose gel and transferred to a positively charged nylon membrane (Roche). A DIG-labeled DNA Molecular Weight Marker was included between each set of samples. Following the optimization guidelines determined above (Figure B), probe 2 was filtered through cellulose acetate and added to the hybridization buffer at a concentration of 25 ng/ml. Our standard protocol was used for hybridization and chemiluminescent detection.

Result and conclusion: The single-copy RFLP is clearly visible in all samples. Background is very low. Therefore, the three-step optimization process described in Figures A and B works and the optimized probe gives good results under actual experimental conditions. Human blood DNA was the chosen target of the customer who sent us the "problem probes" in the first place, so the customer should also be able to get good results from our optimized probe preparation.

C: Suggestions for Improving DIG Labeling and Detection Results

The table below describes ways to get the best results at each stage of the DIG labeling and detection procedure. The influence of each practice on sensitivity and background is also indicated.

For suggestions on improving results of specific DIG labeling and detection procedures, see Chapter 2.

Suggestions	Influence on Background	Influence on Sensitivity
1. General Precautions		
Always work under sterile conditions to eliminate contamination and nucleases. For instance: ▶ Autoclave DIG System solutions. Add Tween 20 to solutions after sterilization. ▶ Use sterile pipette tips. ▶ Filter sterilize solutions containing SDS.	++	++
Use clean incubation trays. Rigorously clean and rinse trays before each use. *For Northern blots, use sterilized glass trays for all washing and detection steps.*	++	++
Wear powder-free gloves when handling membranes.	+	
Handle membrane only on the edges and only with clean forceps.	+	
To become familiar with the DIG System, perform System procedures with the controls included in DIG System Kits.	+	
2. DIG Labeling		
For all labeling methods except PCR labeling, start with as pure DNA template as possible. Ideally, use density gradient purification with CsCl to prepare the template. *Because of the specificity of PCR primers, PCR labeling can produce suitable probes from even impure templates.*	+	+
If you are using cloned sequences as labeling template, try to eliminate as much of the vector from the template as possible before labeling. Labeled vector sequences can lead to troublesome cross-hybridization and nonspecific hybridization signals. ***Example:*** *Digest the vector with a restriction enzyme that cuts as close as possible to the insert to be labeled.*	+	
If large amounts of labeling by-products are formed (*e.g.* secondary amplification products during PCR labeling), reduce the amount of template in the labeling reaction.	+	
If you are using DNA fragments isolated from agarose gels as template, purify the isolated fragment (*e.g.* with the Roche Agarose Gel DNA Extraction Kit or Elutips from Schleicher and Schuell) before using it in the labeling reaction.		+
If using absorbance to determine template concentration, be sure to use the correct absorbance-to-concentration conversion factor for your DNA. ***Example:*** *We assume that, for an oligonucleotide, 1 A_{260} unit = 33 µg oligonucleotide. However, the base composition of the oligonucleotide can significantly affect the absorbance. The actual absorbance of the nucleotide is the sum of the individual contributions of each base.*		+

Suggestions	Influence on Background	Influence on Sensitivity
Addition of the rather bulky DIG-dUTP to DNA generally slows down enzymatic copying of the DNA. Thus, for each type of labeling reaction, there is an optimal DIG-dUTP:dTTP ratio (*i.e.*, one that allows insertion of an optimal density of DIG while slowing the polymerase only minimally). This ratio differs from method to method. In general, use only the DIG-dUTP:dTTP mixtures provided in the labeling kits for a given method. Do **not** substitute mixtures with other ratios.		+
Proteinase K treatment of the labeling reaction can sometimes enhance sensitivity and reduce background. ***Brief procedure:*** Add 0.1 volume stock (20 mg/ml) proteinase K. Incubate for 2 h at 37°C. Use the digested product directly for hybridization.	+	+
It is not usually necessary to remove unincorporated DIG nucleotides from labeled probe before using it in hybridization. ***Exception:*** If you are using the probe for in situ hybridization, you should remove the unincorporated DIG nucleotides before using the probe.		
When producing labeled antisense RNA probes for Northern blot experiments, be sure to use the correct DNA strand for transcription.	+	+
3. Electrophoresis and Blotting		
General electrophoresis tip: For maximum convenience, load a DIG-labeled molecular weight marker on one lane of the gel. The markers will be visible during the detection procedure and allow calculation of molecular weights for bands of interest. *The amount of molecular weight marker needed on the gel will depend on the expected size of your hybridization product. Make sure to load enough marker to produce prominent bands that are about the same size as your hybrid.*		+
Electrophoresis of RNA: For separating RNA samples, you can use gels containing either 2% or 6% formaldehyde. However, we find the 2% formaldehyde gels give sharper bands.		+
Electrophoresis of RNA: Some polylinker regions in plasmids are homologous to portions of the ribosomal 28s and 18s RNA sequences. Therefore, labeled probes may generate specific, but unwanted signals in samples that contain these prominent RNAs. You can prevent this by reducing the amount of ribosomal RNA in your target RNA samples.	+	+
Electrophoresis of RNA: There are many protocols for separating RNA on gels. We recommend using a formaldehyde/MOPS gel, because we have found it to be easy to handle and reliable.		+
Electrophoresis of RNA: RNA samples should be denatured in a formamide/formaldehyde solution. *Glyoxal denaturation of sample followed by separation on a phosphate buffered gel will also work with the DIG System. However, we do not recommend it since we find glyoxal more difficult to use than formamide/formaldehyde. Success depends greatly upon the quality of the glyoxal and on deionizing the glyoxal just before use.*	+	+

Suggestions	Influence on Background	Influence on Sensitivity
3.1. Pretreatment of DNA in the agarose gel before transfer DNA of a certain size or structure must be pretreated with acid or UV light in the gel to ensure efficient transfer. For example, DNA targets larger than 5 kb and supercoiled plasmids must be fragmented before they are transferred. ***Comment*:** If you are only transferring or detecting small (5 kb) DNA targets, you do not need to treat the DNA before transfer. Conversely, for transfer of very large (Mb) fragments or supercoiled plasmid, you may need to use both HCl and UV treatment. The exact conditions need to be determined empirically. Pretreatment (depurination) with acid: Treat the gel with 0.25 M HCl for 10 – 20 min (no more than 10 min for mammalian DNA, up to 20 min for plant DNA). ***Tip*:** Do not pretreat with HCl too long; excessive treatment may degrade the DNA to very small fragments, which will be lost during transfer. Pretreatment with UV light: Use a UV transilluminator to fragment the DNA in the gel. *For every transilluminator, you must determine the irradiation time empirically.*		+
3.2. Denaturation Unless you are using alkaline transfer, denature double-stranded DNA in the gel prior to transfer by treating the gel with 0.5 M NaOH/1.5 M NaCl, 2 × 15 min. Wash the gel briefly in water after the alkali treatment. ***Tip*:** Do not exceed 2 × 15 min incubation in Denaturation Solution if you are using DIG-labeled DNA markers. Prolonged exposure to the alkaline condition will remove the DIG from the markers.		+
3.3. Neutralization Check the pH of the gel before transfer, especially if you are transferring DNA to nitrocellulose membranes. To check the pH of the gel, press a pH strip onto an area of the gel that contains no DNA and then read the strip. *If the pH of the gel is > 9 during the transfer, a nitrocellulose membrane will turn yellow and break apart during hybridization. A nylon membrane will tolerate a higher pH than a nitrocellulose membrane.*		+

Suggestions	Influence on Background	Influence on Sensitivity
3.4. Capillary transfer		
▶ There are many methods for transferring nucleic acids from agarose gels to membranes. For the greatest sensitivity, we find that the preferred transfer method is: capillary blot > vacuum blot > dry blot.		+
▶ We strongly recommend using Roche positively charged Nylon Membranes for transfer. These require no pretreatment and are designed for the DIG System. In contrast, nitrocellulose membrane must be prewetted with distilled water and 20× SSC before the transfer.	+	+
▶ Always handle membranes with extreme care. Always wear powder-free gloves and touch the membrane only with forceps. Handle the membrane by its edges. Do not leave any fingerprints on the membrane.		+
▶ **Special tip for Northern blots:** Presoak gel in sterile (autoclaved) transfer buffer for 10 min prior to transfer. This removes components of the gel running buffer (such as formaldehyde) that may inhibit transfer.		+
! *Ethidium bromide staining does not inhibit transfer of RNA.*		
▶ When preparing the gel-membrane "sandwich", roll a clean pipette gently across the membrane to remove all air bubbles between the gel and the membrane.		+
▶ Place Parafilm around the gel edges to prevent contact of the blotting paper with the Whatman paper and transfer buffer beneath the gel.		+
▶ Make sure the transfer buffer can only flow through the gel, not around the gel. Place the paper towels so they do not touch the Whatman paper beneath the gel or the transfer buffer. Replace the blotting paper when it becomes completely soaked.		+
▶ Use 20× SSC as transfer buffer. Do not use 10× SSC unless very large DNA fragments are to be transferred.		+
▶ Adjust the weight atop the blot "sandwich" according to the size of the gel. We recommend a 200 g weight for a mini-gel and a maximum of 500 g for a 20 × 20 cm gel.		+
▶ After transfer, stain the gel with ethidium bromide to make sure all the nucleic acid has been transferred.		+
3.5. Fixation		
Fix the nucleic acid to the membrane prior to hybridization. Either bake the membrane or use UV light for the crosslinking.	+	+
Baking: For most membranes, bake for 2 h at 80°C. For Roche Nylon Membranes, bake at 120°C for 30 min.		
UV: Use a UV transilluminator and determine the proper irradiation time experimentally. Alternatively, use a commercial crosslinking device		
(*e.g.* a Stratalinker) and use almost any of the standard programs for that device (most will give good results).		
4. Prehybridization and Hybridization		
Remember that RNases can degrade single-stranded RNA bound to the membrane. When handling Northern blots, always work under RNase-free conditions throughout the prehybridization and hybridization procedures.		+

Suggestions	Influence on Background	Influence on Sensitivity
4.1. Prehybridization		
▶ **Do not allow the membrane to dry between prehybridization and hybridization.**	++	
▶ DIG Easy Hyb is the best buffer for all DIG System applications.	+	+
▶ Working concentrations of Blocking Reagent is 1% for DIG-labeled probes. Use a sterile 10% stock solution of Blocking Reagent to prepare the working prehybridization solution.	+	
▶ You may add denatured fish sperm DNA or yeast RNA (50 µg/ml) to the prehybridization solution, but these are not necessary for most applications.		
▶ Be sure to use enough prehybridization solution. For example, if performing the prehybridization in roller bottles, use a minimum of 20 ml prehybridization solution in each bottle.	+	
▶ Prehybridize for at least 30 min at the temperature to be used during hybridization.	+	
▶ The container must be sealed during prehybridization and hybridization to prevent the DIG Easy Hyb buffer from releasing ammonia and changing the pH of the incubations.	+	+
▶ If using sealed bags, shake them gently during the prehybridization.		
▶ The prehybridization buffer can be saved and reused several times.	+	
4.2. Hybridization with probe		
▶ For the lowest possible background with random primed labeled probes, always filter the probe-DIG Easy Hyb mixture through a 0.45 µm cellulose acetate filter before adding it to the membrane. ⚠ *Do not try to filter hybridization buffers that contain blocking solution, since this solution will clog the pores of the membrane.*	+	
▶ Always determine the optimal hybridization conditions empirically for each probe and target.	+	+
▶ Probe concentration is critical. If there is too much probe, the excess may bind nonspecifically to membrane. If there is too little probe, the sensitivity of the reaction will be lower. ❗ *The probe concentrations given in this guide will work for most routine applications. For important experiments, we recommend that you determine the optimal probe concentration in a mock hybridization (see Chapter 5, Part 2 for an example).*	+	+
▶ Buffers and protocols listed in this User's Guide have been optimized to work with the DIG System. However, any established hybridization buffer or protocol may be used with the DIG System.	+	+
▶ DIG-labeled probes are stable for at least 1 year. If stored in hybridization buffer, DIG-labeled DNA probes may even be reused several (generally 3 – 5) times.		
4.2.1. Denaturation of probe		
▶ Always denature DNA probes and RNA probes before adding them to the hybridization solution. ❗ *If you reuse probes (in DIG Easy Hyb), denature the probe solution before use by heating it to 68°C for 10 min. (Do not boil!)*		+
▶ Denaturation of oligonucleotides is only necessary when the sequence is expected to form secondary structure.		
▶ If the hybridization solution contains formamide, use only deionized formamide.	+	+

Suggestions	Influence on Background	Influence on Sensitivity
4.2.2. Hybridization in roller bottles		
▶ Use at least 6 ml hybridization solution per roller bottle. If necessary, increase the volume.	+	+
▶ Carefully monitor the hybridization temperature inside the roller bottle. Note that the temperature of the incubation oven may not necessarily be the same as that inside the bottle. To check the temperature before hybridization, fill the bottle with water and place a thermometer in the water.	+	+
4.2.3. Hybridization in sealed bags		
▶ Use at least 3.5 ml hybridization solution per 100 cm^2 membrane.	+	+
▶ This volume may be increased to 5 ml per 100 cm^2 if enough probe is available.		
▶ **Remove all air bubbles in bag before sealing.**	+	+
▶ Check that the bag is totally sealed on all sides. Leakage during hybridization may allow the membrane to dry.	+	+
▶ Gently shake the sealed bag in a water bath set at the correct hybridization temperature. The membrane should lie flat on the bottom of the bath.	+	+
4.2.4. Special tips for hybridization with tailed oligonucleotides		
▶ If the probe is a tailed oligonucleotide, add 0.1 µg/ml poly(dA) to the prehybridization and hybridization solutions to prevent nonspecific hybridization.		+
▶ Addition of 5 µg/ml poly(A) to the prehybridization solution can increase blocking efficiency.		+
4.3. Special tips for Northern blot hybridization		
▶ Always work under sterile, RNase-free conditions. Membrane-bound single-stranded RNA and double-stranded RNA hybrids can be degraded by specific RNases.		
▶ The best hybridization buffer for Northerns is DIG Easy Hyb. However, an acceptable alternative is the high SDS buffer listed in Section 6 of Chapter 1. If using any buffer except DIG Easy Hyb, be sure to include 50% formamide in the buffer.		
▶ Use RNA probes whenever possible; they are more sensitive than DNA probes for detecting RNA targets.		
▶ If you are using RNA probes, we recommend that you also use the ready-to-use buffers in the Wash and Block Buffer Set (Cat. No. 11 585 762 001) to avoid unsatisfactory results caused by RNase contamination in "homemade" buffers (*e.g.,* Blocking Solution made from a dry powder).		
▶ Most labeled RNA probes are completely free of vector sequences. However, depending on the restriction enzyme you used to prepare template, remember that the probe could still contain some multilinker sequences that could lead to unwanted background.		
▶ If you are using RNA probes, you can reduce the background by adding an RNase A wash after the last stringent wash. **Procedure:** Prepare a wash solution containing 100 µg/ml RNase A in 10 mM Tris/5 mM EDTA/300 mM NaCl, pH 7.5. Incubate the blot in this RNase A wash solution for 30 – 60 min at room temperature. ⚠ *Some RNase A preparations may contain double-strand-specific RNases that can degrade hybrids on the membrane. To detect these ds-specific RNases, incubate the RNase A solution with MS2 RNA, then analyze the product on a denaturing agarose gel. Compare the product with untreated MS2 RNA. MS2 RNA is partially double-stranded. If the RNase A solution is free of ds-specific activity, it will leave the double-stranded regions of MS2 RNA intact.*		
▶ We do not recommend storing and reusing hybridization solutions containing RNA probes.		

Suggestions	Influence on Background	Influence on Sensitivity
4.4. Special tips for colony and plaque hybridizations		
▶ All cellular debris **must** be removed from the membrane before starting hybridization. For details, see Chapter 2, Section 6.1.	+	+
▶ The probe preparation must not contain any vector sequences.	+	+
4.5. Post-hybridization Washes		
▶ Optimize the wash conditions experimentally for your particular application. *The stringent washes listed in this guide are appropriate for probes that are 100% homologous to target DNA and that contain approx. 50% GC.*	+	+
▶ For genomic hybridizations, we recommend 0.5x SSC in the washes. However, it may be necessary to increase the stringency (*e.g.,* by using 0.1x SSC).	+	+
▶ Always shake the tray containing the washes vigorously.	+	
▶ Prewarm the wash solutions to the wash temperature before adding it to the blot.	+	
▶ Do not use bottles for washes or antibody incubation steps.	+	
▶ Use enough washing solution to fully submerge, not just cover the blot membrane. Be sure there is enough wash solution to keep membranes from overlapping or sticking together during the incubations.	+	
5. Immunological Detection		
▶ Work under sterile conditions.	+	
▶ Always use freshly cleaned trays.	+	
▶ Shake membranes during all the steps of the detection procedure except the color/chemiluminescent development step.	+	
▶ Store the antibody at 4°C.	+	
▶ Prepare a fresh dilution of the antibody directly before use. To prepare a fresh antibody solution centrifuge the antibody in the original vial for at least 5 min at 10.000 rpm and pipett the antibody from the supernatant.	+	
▶ Prepare a fresh dilution of the antibody directly before use. To prepare a fresh antibody solution centrifuge the antibody in the original vial for at least 5 min at 10.000 rpm and pipett the antibody from the supernatant.	+	
▶ You may prolong the blocking and washing steps, but do not prolong the antibody incubation.	+	
▶ The concentration of the Blocking Reagent may be increased to 5% if nonspecific background is a problem.	+	
▶ Always transfer the blot to a freshly washed tray for the steps after the antibody incubation.	+	
▶ If using chromogenic substrate, prepare a fresh dilution of it directly before use.	+	
▶ If working with chemiluminescent substrate, you should dilute the antibody 1:10 000. For reactions with CDP-*Star*, you may even be able to use a 1:20 000 dilution of the antibody.	+	
▶ When handling chemiluminescent substrates, use sterile technique and avoid phosphatase contamination.	+	
▶ Perform the chromogenic incubation in the dark without shaking. *It is not necessary to work in the dark when applying chemiluminescent substrates to the membrane.*	+	

Suggestions	Influence on Background	Influence on Sensitivity
▶ You can treat a single blot sequentially with both a chemiluminescent and a chromogenic substrate. ***Procedure*:** After chemiluminescent detection is complete, wash the chemiluminescent substrate off the membrane for 5 min with Detection Buffer. Then add the color substrate. Background from the back of the membrane will not be a problem in this case.	+	
▶ Different types of X-ray film have different sensitivity. For best results, use Roche Lumi-Film, Kodak XAR, or DuPont Cronex 4.		+
▶ Dilutions of CSPD and CDP-*Star* can be reused one or two times within a month. Between uses, filter the substrate, add sodium azide, and store the filtered solution in the dark at 4°C. Avoid contaminating the stored solution.		
6. Stripping and Reprobing		
Do not use a higher concentration of NaOH or a higher incubation temperature than those recommended in Chapter 3. ***Example*:** The stripping conditions recommended in some systems (*e.g.,* 0.4 M NaOH at 65°C) will remove substantial target DNA from the membrane.		+
Always store the Stripping Buffer in plastic bottles; never use glass bottles. The NaOH will react with the silica in the glass, forming salts. The stored solution will therefore have a reduced concentration of NaOH and may not be effective in removing the probe.	++	+
Never let the membrane dry at any time before you strip it.		+
Store stripped membrane in 2× SSC.		+

D: Preparation of Solutions

1. Solutions for DNA and RNA Labeling

The reagents required for labeling depend on the labeling method chosen. For a complete list of reagents required for each labeling method, see the following sections of Chapter 3:

For this labeling method...	Reagents are listed in...	Starting on page
Random primed labeling of DNA	Section 2.1.1, Chapter 3	58
PCR labeling of DNA	Section 2.2.1, Chapter 3	64
Transcriptional labeling of RNA	Section 2.3.1, Chapter 3	75

2. Solutions and Buffers for DNA/Southern Blotting and Hybridization

Water

Always use autoclaved, double distilled H_2O for all Southern blotting/hybridization solutions and buffers.

TE buffer

10 mM Tris-HCl

1 mM EDTA

Adjust pH to 8.0 (25°C).

DNA probe dilution buffer (for estimate of probe yield)

TE buffer containing 50 µg/ml herring sperm DNA (included in the DIG-High Prime Labeling and Detection Starter Kit II)

DNA gel running buffer

89 mM Tris-borate

2 mM EDTA

Adjust pH to 8.3 (25°C).

Depurination solution

0.25 M HCl

Denaturation solution (for Southern transfer or colony/plaque hybridization)

0.5 M NaOH

1.5 M NaCl

Neutralization solution 1 (for Southern transfer)

0.5 M Tris-HCl, pH 7.5

1.5 M NaCl

Neutralization solution 2 (for colony/plaque hybridization)

1.0 M Tris-HCl, pH 7.4

1.5 M NaCl

DIG Easy Hyb hybridization buffer

Option 1: Purchase ready-to-use, DNase- and RNase-free DIG Easy Hyb solution as an individual reagent (Cat. No. 11 603 558 001). Use as supplied for prehybridization or add the appropriate amount of labeled DNA probe for hybridization.

Option 2: Purchase DNase- and RNase-free DIG Easy Hyb granules as an individual reagent (Cat. No. 11 796 895 001) or as part of the DIG-High Prime Labeling and Detection Starter Kit II (Cat. No. 11 585 641 910).

Add 64 ml sterile, double distilled water (in two aliquots) to 1 portion of granules. Stir the solution for 5 min at 37°C until granules dissolve. Use reconstituted DIG Easy Hyb without further additives for prehybridization or add the appropriate amount of labeled DNA probe for hybridization.

20× SSC (Cat. No. 11 666 681 001)

3 M NaCl

0.3 M sodium citrate

Adjust pH to 7.0 (20°C) and autoclave.

Low stringency wash buffer

2× SSC (1:10 dilution of stock 20× SSC)

0.1% SDS (1:100 dilution of stock 10% SDS)

High stringency wash buffer

0.5× SSC (1:40 dilution of stock 20× SSC)

0.1% SDS (1:100 dilution of stock 10% SDS)

Probe stripping solution (for DNA probes labeled with alkali-labile dUTP)

0.2 M NaOH

0.1% SDS (1:100 dilution of stock 10% SDS)

Membrane wash buffer (after probe stripping)

2× SSC (1:10 dilution of stock 20× SSC)

3. Solutions and Buffers for RNA/Northern Blotting and Hybridization

Water

Always use autoclaved, DMDC- or DEPC-treated, double distilled H2O for all Northern blotting/hybridization solutions and buffers. DMDC-/DEPC-treated water is RNase-free.

Preferred treatment: Dissolve 1% (*e.g.*, 1 g per 100 ml) dimethyl-dicarbonate (DMDC) in a 50% ethanol/water mixture. Mix double distilled H_2O 1:10 with the stock 1% DMDC solution (final concentration, 0.1% DMDC). Incubate for 30 min at room temperature, then autoclave the treated water.

You can also use DEPC (diethylpyrocarbonate) to treat the water. However, we prefer DMDC, since it is less toxic than DEPC.

RNA probe dilution buffer (for estimate of probe yield)

Mix DMDC- or DEPC-treated water, 20x SSC, and formaldehyde in the ratio 5:3:2.

Always treat water and 20x SSC with DMDC or DEPC before use (to destroy RNases).

10× MOPS buffer

200 mM morpholineopropanesulfonic acid (MOPS)

50 mM sodium acetate

20 mM EDTA

Adjust pH to 7.0 with NaOH. Autoclave.

The solution will turn yellow after it is autoclaved. This will not affect its performance.

Dilute 1:10 to make RNA gel running buffer.

Deionization of formamide

Add 50 g AG 501-X8 ion-exchange resin to 500 ml formamide. Stir slurry slowly for 30 min on a magnetic stirrer. Remove the resin by filtration and store the filtered, deionized formamide at 20°C.

RNA loading buffer

Just before use, prepare a solution containing the following:

100% formamide, deionized	250 µl
37% (w/v) formaldehyde	83 µl
10× MOPS buffer	50 µl
2.5% bromophenol blue	10 µl
100% glycerol, RNase-free	50 µl
DMDC-/DEPC-treated H_2O	
	To make 500 µl total volume (requires approx. 57 µl)

DIG Easy Hyb hybridization buffer

Purchase DNase- and RNase-free DIG Easy Hyb (Cat. No. 11 603 558 001), ready-to-use or DIG Easy Hyb granules as an individual reagent (Cat. No. 11 796 895 001) or as part of the DIG-High Prime Labeling and Detection Starter Kit II (Cat. No. 11 585 641 910).

Just before use, add 64 ml sterile, DMDC-/DEPC-treated, double distilled water (in two aliquots) to 1 portion of granules. Stir the solution for 5 min at 37°C until granules dissolve. Use reconstituted DIG Easy Hyb without further additives for prehybridization or add the appropriate amount of labeled RNA probe for hybridization.

Low stringency wash buffer

2× SSC (1:10 dilution of stock 20× SSC)

0.1% SDS (1:100 dilution of stock 10% SDS)

High stringency wash buffer

0.1× SSC (1:200 dilution of stock 20× SSC; see Section 2 above for 20× SSC)

0.1% SDS (1:100 dilution of stock 10% SDS)

Probe stripping solution (for RNA probes)

50% formamide

50 mM Tris-HCl, pH 7.5

5% SDS

Membrane wash buffer (after probe stripping)

2× SSC (1:10 dilution of stock 20× SSC)

4. Required Solutions and Buffers for Detection

For detection of RNA hybrids on Northern blots, always use DMDC- or DEPC-treated water to make all detection reagents (see Section 3 above). Observe RNase-free conditions when making the reagents.

Maleic Acid Buffer

Purchase sterile, nuclease-free stock buffer (10× concentrated) as part of the DIG Wash and Block Set (Cat. No. 11 585 762 001).

To make working Maleic Acid Buffer, dilute the stock buffer 1:10 with sterile, double distilled water.

Washing Buffer

Purchase sterile, nuclease-free stock buffer (10x concentrated) as part of the DIG Wash and Block Set (Cat. No. 11 585 762 001).

Before use, shake the stock solution to resuspend the buffer components. To make working Washing Buffer, dilute the stock buffer 1:10 with sterile, double distilled water.

Blocking Solution

Purchase sterile, nuclease-free, stock solution (10x concentrated) as part of the DIG Wash and Block Set (Cat. No. 11 585 762 001), the DIG-High Prime Labeling and Detection Starter Kit II (Cat. No. 11 585 641 910), or the DIG Northern Starter Kit (Cat. No. 12 039 672 910).

To make working (1%) Blocking Solution, dilute the stock solution 1:10 with working Maleic Acid Buffer just before use.

Detection Buffer

Purchase sterile, nuclease-free stock buffer (10× concentrated) as part of the DIG Wash and Block Set (Cat. No. 11 585 762 001).

To make working Detection Buffer, dilute the stock buffer 1:10 with sterile, double distilled water.

Chemiluminescent substrate solution

Option 1: Purchase ready-to-use CDP-*Star* as an individual reagent (Cat. No. 12 041 677 001) or as part of the DIG Northern Starter Kit (Cat. No. 12 039 672 910). Use as supplied.

Option 2: Purchase ready-to-use CSPD as an individual reagent (Cat. No. 11 755 633 001) or as part of the DIG-High Prime Labeling and Detection Starter Kit II (Cat. No. 11 585 641 910). Use as supplied.

Color substrate solution

Option 1: Purchase NBT/BCIP stock solution as an individual reagent (Cat. No. 11 681 451 001) or as part of the DIG-High Prime Labeling and Detection Starter Kit I (Cat. No. 11 745 832 910). Just before use, add 200 µl of the stock solution to 10 ml of Detection Buffer to make Color Substrate Solution.

Option 2: Purchase NBT/BCIP tablets as an individual reagent (Cat. No. 11 697 471 001). Just before use, add one tablet to 10 ml of sterile, double distilled water to make Color Substrate Solution.

AP inactivation buffer

50 mM EDTA

Adjust pH to 8.0 (20°C).

5. Other Solutions and Buffers

N-lauroylsarcosine stock solution

Dissolve 10% (w/v) N-lauroylsarcosine in water.

Filter through a membrane (0.2 – 0.45 µm).

High SDS hybridization buffer

7% SDS

50% formamide, deionized

5× SSC

0.1% N-lauroylsarcosine

2% Blocking Solution [1:5 dilution of stock (10%) Blocking Solution; see Section 4 above.]

50 mM sodium phosphate, pH 7.0

To prepare 500 ml of high SDS hybridization buffer, combine the following stock solutions in the order given below:

100% formamide, deionized	250 ml
30× SSC	83 ml
1 M sodium phosphate, pH 7.0	25 ml
10% Blocking Solution	100 ml
10% N-lauroylsarcosine	5 ml

Pour the resulting solution into an Erlenmeyer flask that contains 35 g SDS.

⚠ *Do not inhale SDS. Heat and stir the solution until the SDS dissolves. Adjust the total volume of the solution to 500 ml with sterile, double distilled water.*

Store the solution at room temperature, but heat to 65°C before use.

Standard hybridization buffer

5× SSC

0.1% N-lauroylsarcosine

0.02% SDS

1% Blocking Solution [1:10 dilution of stock (10%) Blocking Solution]

Standard hybridization buffer + 50% formamide

5× SSC

50% formamide, deionized

0.1% N-lauroylsarcosine

0.02% SDS

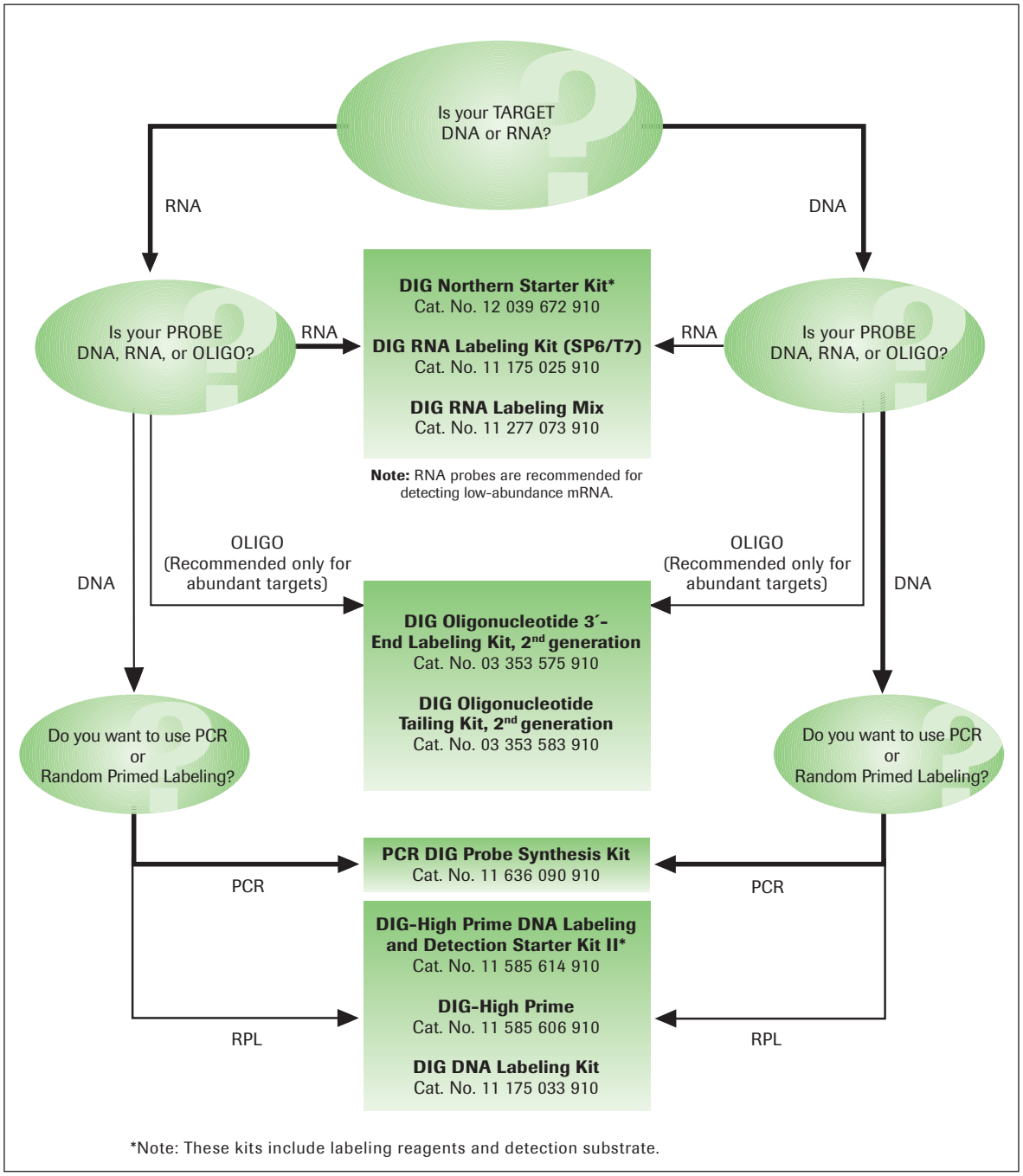
2% Blocking Solution [1:5 dilution of stock (10%) Blocking Solution]

E: DIG Application Guide

1. Determine your labeling method

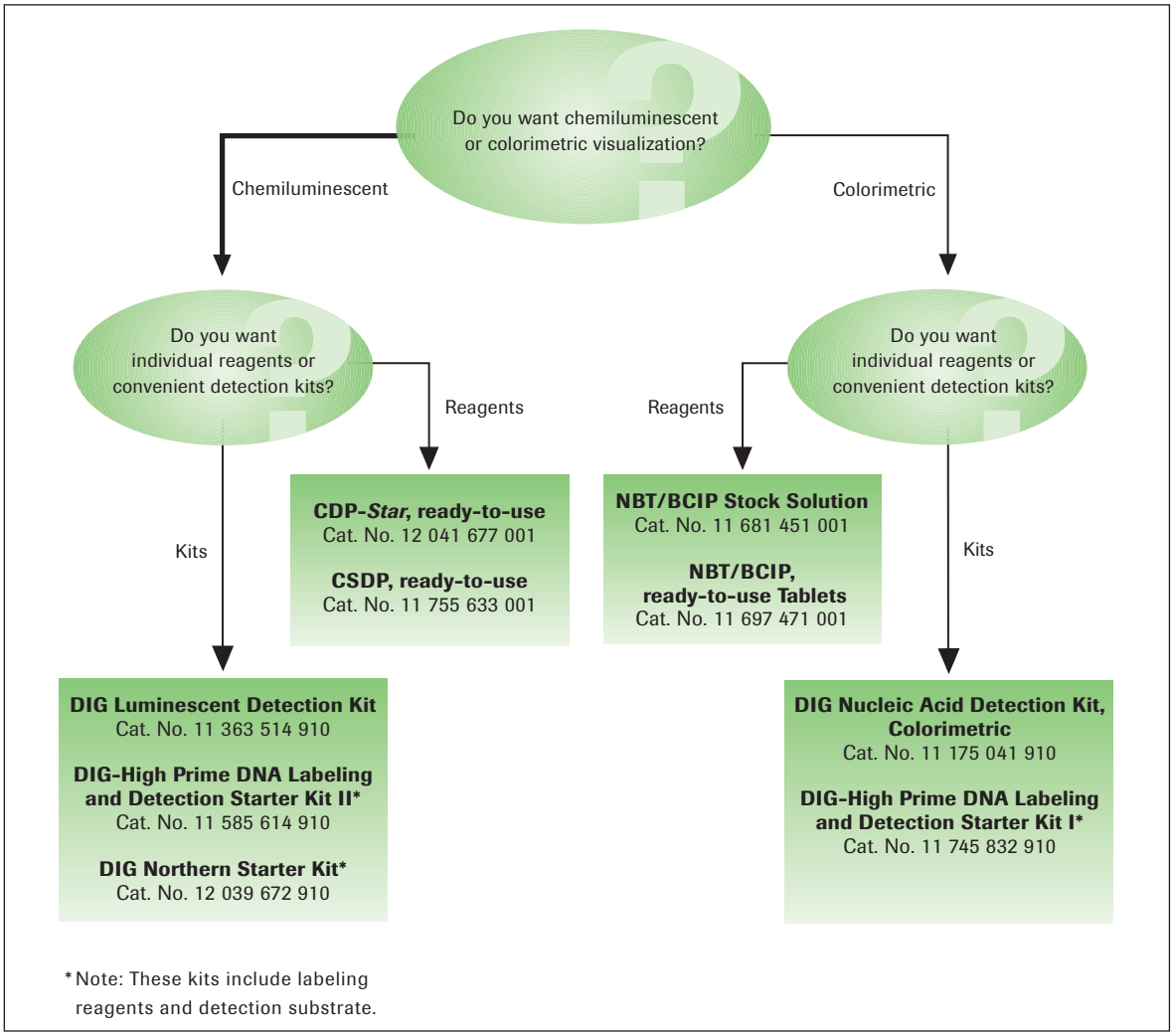
Step 1

(Heavier arrows indicate recommended pathways)



2. Determine your detection method

Step 2



*Note: These kits include labeling reagents and detection substrate.

3. Add other Roche products that will help you get the best results

Step 3

Definitely!

Anti-Digoxigenin-AP, Fab fragments
Cat. No. 11 093 274 910

DIG Wash and Block Buffer Set
Cat. No. 11 585 762 001

DIG Easy Hyb
Cat. No. 11 603 558 001 (solution)
Cat. No. 11 796 895 001 (granules)

Nylon Membranes (positively charged)
Cat. No. 11 209 299 001 (10 x 15 cm)
Cat. No. 11 209 272 001 (20 x 30 cm)
Cat. No. 11 417 240 001 (0.3 x 3 m)

Nylon Membranes for Colony/Plaque Hybridization
Cat. No. 11 699 075 001 (82 mm diameter)
Cat. No. 11 699 083 001 (132 mm diameter)

Hybridization Bags
Cat. No. 11 666 649 001

DNA Molecular Weight Markers, DIG-labeled
Cat. No. 11 218 590 910 (DNA MWM II, DIG-labeled)
Cat. No. 11 218 603 910 (DNA MWM III, DIG-labeled)
Cat. No. 11 218 611 910 (DNA MWM VI, DIG-labeled)
Cat. No. 11 669 940 910 (DNA MWM VII, DIG-labeled)
Cat. No. 11 449 451 910 (DNA MWM VIII, DIG-labeled)

RNA Molecular Weight Markers, DIG-labeled
Cat. No. 11 526 529 910 (RNA MWM I, DIG-labeled)
Cat. No. 11 526 537 910 (RNA MWM II, DIG-labeled)
Cat. No. 11 373 099 910 (RNA MWM III, DIG-labeled)

Lumi-Film
Cat. No. 11 666 657 001 (20.3 x 25.4 cm = 8 x 10 inches)
Cat. No. 11 666 916 001 (18 x 24 cm = 7.1 x 8.4 inches)

The pack inserts of all listed products can be downloaded from our website at http://www.roche-applied-science.com

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