TALLER INTERNACIONAL SOBRE " PCR Y SECUENCIACION DE ADN "

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Corpoica

SUBDIRECCION DE INVESTIGACION ESTRATEGICA

Programa de Entrenamiento Avanzado

Corporación Colombiana de Investigación Agropecuaria

- C O R P O I C A -

TALLER INTERNACIONAL SOBRE PCR Y SECUENCIACION DE ADN

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The Basics of Molecular Biology

Contents

The primer was prepared to explain some of the basics of molecular biology. It is not intended to be a comprehensive account of everything that happens in a molecular biology laboratory. If you have any specific questions or comments about this information or other molecular biology techniques, please feel free to contact the following:

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About NBI

NBI (National Biosciences, Inc.) provides both biotechnology software and research services for the biotech/bioscience industry.

Software

NBI develops, distributes, and supports several premier software packages for both the Mac and PC platforms. Our product line includes programs for PCR® analysis, genetic pedigree drawing and analysis, human genome mapping, multiple sequence alignment, DNA and protein sequence analysis, motif searching, and molecular modeling.

Many of our software products are developed internally, and we also carry a wide range of products from other developers.

All of NBI's software customers receive technical support from our scientific staff via our toll-free number or E-mail.

NBI offers multi-user and network licenses, in addition to DOS®, WINDOWS™ and MAC® systems. We also have a discount policy for non-profit organizations.

NBI markets directly and through a number of North American and international sales organizations, including representatives in Canada, Norway, Sweden, Italy, Brazil, Australia, Japan, Taiwan, Israel, and Korea. NBI is headquartered in Plymouth, Minnesota.

Services

NBI has a full-service custom laboratory. We provide custom DNA and RNA synthesis services, phosphorothioates, oligo design services, custom DNA sequencing, gene synthesis, cloning, and library screening/construction services. We also provide specialty services and work individually with customers requiring these for their particular research projects.

Our staff includes synthesis chemists, molecular biologists, and geneticists.

Our custom synthesis services are recognized internationally. We have the most complete quality control system anywhere for synthesis services. With each synthesis service order, NBI returns, via our *FAXBACK* system, an order confirmation verifying the sequence and other particulars, plus an on-line analysis report assuring that there are no potential design problems with the order.

We are the only synthesis service provider with on-line analysis, wherein customers may redesign their orders, if needed, prior to synthesis. Our on-line analysis system is based on NBI's own *OLIGO Primer Analysis Software*. With the order, NBI includes a comprehensive synthesis report providing a broad range of scientific and QC data on each oligonucleotide.

OLIGO Primer Analysis Software General Information

PCR, Sequencing, and Hybridization Applications

OLIGO is a multi-functional program that searches for and selects oligonucleotides from a sequence file for polymerase chain reaction (PCR), sequencing, site-directed mutagenesis, gene synthesis, and various hybridization applications. It calculates hybridization temperature and secondary structure of oligonucleotides based on nearest-neighbor thermodynamic values.

Essential Qualities for Optimal Primers & Probes

There are three essential qualities to optimal PCR primers, sequencing primers, and hybridization probes. They should be highly specific for the intended target sequence and not hybridize to other regions within the template. Because sequencing conditions are typically not very stringent and the formation of primer-template duplexes with imperfect homology is significant, specific primers are essential. PCR or sequencing results are generally poor because of high background if there is a base-pairing between the primer 3' terminus and sites other than the intended target in the template.

Another important requisite of primers is that they do not form dimers and hairpins during reactions. Oligonucleotides that form dimers or hairpins function poorly in site-directed mutagenesis and in sequencing reactions, especially if double stranded DNA is used. Dimer and/or hairpin-forming primers are particularly troublesome when 3' termini are tied up; this can cause internal primer extension, which eliminates the primer from the reaction and may contribute to false priming.

Finally, primers and probes should form stable duplexes with a template under the appropriate conditions. GC-rich regions are more stable than AT-rich regions, and the duplex stability depends upon the sequence order. Consequently, this affects the outcome of PCR or sequencing reactions, and also site-directed mutagenesis.

The OLIGO program is designed specifically to search for and list the position, sequence, and other data of all oligonucleotides (or primer pairs) that optimally meet these major requirements, plus several other criteria outlined in this guide.

About the OLIGO Program

Technical Specs

The following are the OLIGO 5.0 system requirements and technical specifications:

	Minimum	Suggested
Computer:	386/16 Mhz	486/33 Mhz or higher
Operating System:	WINDOWS 3.1	WINDOWS 3.1 or higher
Memory:	4 MB of RAM	8 MB of RAM or higher
Monitor:	VGA	SVGA or higher

Program Information

Program Size:	1 MB
Number of Disks:	2 HD Disks
Acceptable Sequence File Formats:	Text, EMBL, GenBank, GCG, Entrez

Program Features

The OLIGO program is the premier package for searching, selecting, and analyzing oligonucleotides. In this demo, you can review the following features and capabilities:

- Oligonucleotide Databases
- PRIMEFORM Oligonucleotide Ordering Software
- Hybridization Time Calculations
- Concentrations Calculations
- Optimal LCR Primer Selection
- Restriction Sites and Palindromes Searches
- Template Secondary Structure Searches
- Cross-compatible Multiplex Primer Searches
- Amino Acid Back Translation
- Sequencing Editing Capabilities
- Priming Efficiency Calculations

Program Features Disabled in This Demo

In order to provide you with the best understanding of the power of OLIGO, we have made this demo interactive — you can try out most features, but with sample data only. The following options are disabled or modified in this demo:

- The *File — Open* option is disabled. The demo automatically loads the DNA sequence CBP.SEQ as a sample file.
- Keyboard entry in the "Edit" Window is randomized.
- The *File — Open Database* feature is disabled. Review the database capabilities using *File — New Database*.
- PRIMEFORM is fully functional, but is only an NBI ordering form.
- The oligonucleotide database *Edit* feature is disabled.
- Context-sensitive help is not included in the demo due to space limitations.

Getting Started With the OLIGO Demo Disk

Loading the Demo Disk to Your Hard Drive

You need to load the OLIGO demo disk to your hard drive and must be familiar with Windows to use the program. To load the program to your hard drive:

1. Start Microsoft Windows.
2. Insert the OLIGO Demo Disk in your floppy drive, usually drive A.
3. From the *Program Manager* application, select the *File* menu and choose *Run*.
4. Enter **a:\setup**. A progress bar indicating that the setup is initializing appears.
5. You must enter a path or accept the default on the "Installation" screen. Click "OK."
6. Click "OK" on the "Installation Complete" screen.
7. Once the installation is complete, the OLIGO icon appears. Double click on this to call up the OLIGO demo program.
8. Click "OK" on the "Welcome" screen.

The Main Screen

The main screen of OLIGO appears just after you click "OK" on the "Welcome" screen. This main screen consists of two windows: the "Melting Temperature" window and the "Internal Stability" window.

The Melting Temperature Window

The Melting Temperature window displays a segment of the active DNA/RNA sequence file and a T_m plot of the oligonucleotides with a default length of 21 nt within the segment. The length of the segment displayed will vary, depending upon the monitor resolution. Each point on the T_m plot represents the melting temperature of a 21-mer oligonucleotide, called the Current Oligo, the oligonucleotide sequence currently available for analysis. To view a T_m plot of the entire file, select the "zoom out" feature from the T_m Window icon.

The T_m (or ΔG) of any oligonucleotide on the plot is read on the y-axis by using the cross-hairs cursor.

The horizontal line bisecting the T_m plot represents the average T_m (or ΔG) of all the 21-mer oligonucleotides in the sequence file. Below the T_m plot is the nucleotide sequence (both strands) in lower case letters. Corresponding amino acids appear in color below the lower primer.

The Internal Stability Window

The "Internal Stability" window appears below the "Melting Temperature" window. This window displays an oligonucleotide internal stability (ΔG) plot that can be used with high confidence to predict the specificity of the displayed oligonucleotides intended for PCR or sequencing reactions.

Working with the OLIGO Demo

Demo Disk Features

The OLIGO demo program shows OLIGO's power to search, select, and analyze oligonucleotides for the most common laboratory applications using the CBP.SEQ file, the sample DNA sequence file available in the demo version.

There are several examples of OLIGO features following the search selection criteria table in this guide to help you learn how OLIGO can help in your research.

OLIGO Icons

UPPER

LOWER

The Upper and Lower (red and blue) icons save the Current Oligo as the Upper Primer (+ strand) or Lower Primer (- strand), respectively. The length of the current oligo is displayed in the title bar (default = 21 nt).

This icon calls up a dialog box permitting the movement of the current oligo to a specified base position on the sequence file.

This icon centers the Current Oligo in the window.

This moves the Current Oligo to the beginning of the sequence file.

This icon moves the Current Oligo to the end of the sequence file.

The horizontal scroll bar represents the entire length of the sequence file. Clicking and dragging the scroll bar box along the scroll bar moves the Current Oligo to an approximate position on the sequence file. Clicking to the left or right of the scroll bar box moves the Current Oligo one window length toward either end of the sequence file.

Using Reset & Verifying Parameters

As you use the OLIGO demo, note that you can "wipe the slate clean" of data positions by using *Reset* from the *File* menu and selecting "Data." If you want to duplicate the exact results of the examples in this guide, however, select *Reset* and "Original Defaults" and *Reset* "Data" to make sure that no program parameters and other settings have changed, and to erase previous data. "Original Defaults" resets all non-search parameters. "Data" resets data, including Upper and Lower Primers.

Closing Windows & Exiting

When you have completed a search, close the windows by selecting *Close* from the system-menu box. This is the small horizontal box to the far left of the window title bar.

To exit OLIGO, select *Exit* from the *File* menu, or press <Alt><F4>.

Definitions Used in OLIGO

Current Oligo

The Current Oligo is the oligonucleotide displayed in upper case letters and underlined in the main window. It includes both the + and - strands and is updated with each position change on the active sequence. The Current Oligo can be saved as an Upper or Lower Primer and/or analyzed using most options from the *Analyze* menu.

Free Energy

ΔG stands for free energy, and is a measurement of nucleic acid duplex stability. A DNA duplex is stable when its ΔG value is negative. The ΔG depends on salt concentration, temperature, and other factors.

Internal Stability

Internal stability refers to the stability of sub-sequences within an oligonucleotide, specifically to 5 base segments (pentamers). The stability of these internal pentamers is expressed in kcal/mol (ΔG values).

Lower Primer/ Upper Primer

The Lower Primer is a negative strand oligo that has been selected for analysis, generally as a PCR or sequencing primer. It can be manually selected by you or automatically selected by OLIGO, as in a search for PCR primer pairs. The Upper Primer functions the same, but is a positive strand primer.

n OD_{260}

This is the amount of nucleic acid that would give absorption equal to *n* if dissolved in a 1 ml buffer (pH 7.0) and measured in a cuvette with a path length of 1 cm. Approximately 30 OD_{260} = 1 mg of single stranded DNA.

Priming Efficiency

The priming efficiency number is a formulation unique to the OLIGO program that quantifies the likelihood that a given oligonucleotide will prime at a given site on the template. The priming efficiency calculation is derived from an algorithm that considers duplexes and mismatches and their distance from the 3'-end.

Td — Filter Dissociation Temperature

T_d is the temperature at which 50% of the nucleic acid probe retained on a hybridization filter after five minutes of incubation using 1 M salt and 100 pM of oligos (calculated by nearest neighbor method). $T_d = T_m - 7.6°$

T_m

T_m is the temperature at which 50% of nucleic acid molecules are in duplex (and 50% denatured). For the default T_m calculations, 1 M salt (Na+ or K+, neutral pH) and 0.6 pM nucleic acid concentrations are used in the OLIGO program to provide T_m values based on the nearest neighbor methods that are in line with, but four times more accurate, than the "2xAT+4xGC" method.

OLIGO Examples

Following are several step-by-step examples that will introduce you to some of OLIGO's most powerful features. As you go through the examples, select *Reset* from the *File* menu and choose "Original Defaults" and "Data" to set the program back to the original parameters and settings before each example.

Example 1: Moving Across The Sequence

There are three ways to select a Current Oligo position in the program. Each method is described here.

Method 1 — Scrolling and Clicking

1. Move the scroll bar on the bottom of either window to position the graph in the desired location.
2. Using the mouse, position the cross-hair tool on the position you want and click.
3. OLIGO shifts the graph on both windows and updates the information.

Method 2 — Choosing from the Menu

1. Pull down the *Select* menu from the main menu bar.
2. Choose the *New Current Oligo Position* function from the *Select* menu, or press <F10>.
3. Enter the desired position number in the dialog box.
4. OLIGO shifts the graph on both windows and updates the information.

Method 3 — Using the Icons

1. Click on the far left icon (circle with cross-hairs) at the bottom of either window.
2. Enter the desired position number in the dialog box.
3. OLIGO shifts the graph on both windows and updates the information.

Example 2: A Quick PCR Primers Search

This example takes you through a quick search for a pair of optimal PCR primers. To run this search:

1. Chose the *Primers and Probes* option from the *Search* menu, or press <F3>.
2. Check that the "Compatible Pairs" button under "PCR Primers" in the dialog box is checked.
3. Click on the "Search Ranges" button and set the search range to **800-1000** for the positive strand and **1400-1600** for the negative strand. Click "OK."
4. Click "OK" to start the search.
5. When the search is complete, check on the "Search Status" window to make sure that the final search stringency is acceptable. OLIGO automatically reduces search stringency if it does not find any compatible pairs.
6. Click on the "Primer Pairs" button at the bottom of the window.
7. There are several sort options on the "Primer Pairs" window. For this example, click on the circle above "Product Length" to sort by product length.
8. Click on any primer pair to view the "PCR" data for the selected pair.
9. Choose *Selected Primers* from the *Window* menu to view the primer sequence and other data.

Example 3: A Detailed PCR Primers Search

This example takes you through a comprehensive search for a pair of optimal PCR primers that amplify a product of approximately 500 bases and include the nucleotide subsequence between positions 600 and 800. This search takes about 45 seconds on a 486/50Mhz computer. To run this search:

1. Choose *Reset* from the *File* menu and select "Data" to reset the parameters.
2. Choose the *Search — Primers and Probes* options from the *Search* menu on the main menu bar, or press <F3>.
3. Click on the button next to "Compatible Pairs," if it isn't marked already.
4. Make sure that the boxes for "GC Clamp" and "Eliminate False Priming" are checked.
5. Click the "Continue False Priming Search in Other Files" box to call up the "False Priming Search in Other Files" window.
6. Double-click on the "Freqseq" directory and then on the "Humanfr.seq" file. Then click the "Add" button to add this file to be checked for false priming. Any oligo on the active sequence will be eliminated if it contains reiterative human sequence on the 3'-end.
7. Click "Done."
8. Click the *Search Ranges* button.

OLIGO 5.0 Oligonucleotide Search Selection Criteria			
Performance Problem	**Application**	**Design Remedy**	**Selection Feature**
Background due to false priming in genomic or other DNA samples with much unknown sequence.	PCR, Sequencing	Select oligonucleotides with high specificity.	*3' Stability Window* — Determine stability (ΔG) of the 3' ends of oligonucleotides; select only those with low or moderate stability.
Multiple PCR product bands due to false priming within and near the intended amplification region.	PCR, Cycle Sequencing	Select only oligonucleotides that will not false prime within and near the PCR product on the sequence file.	*False Priming Check* — Determine the propensity of false priming using stability (ΔG) calculations; select oligos with no strong priming affinity to any region on the active sequence file.
Background due to false priming and much of the sample is known but scattered over many sequence files.	Sequencing, PCR	Select oligonucleotides which will not false prime on known sequence outside the active sequence file.	*False Priming Check Against Other Sequence Files* — Check for false priming sites in all the sequence files selected by the user.
High background due to false priming in repetitive sequence files, such as ALU.	PCR, Sequencing	Select oligonucleotides which will not false prime in repetitive sequence files.	*False Priming Check Against Repetitive Sequence Database File* — Check for false priming sites in repetitive sequence files selected by the user.
Very low efficiency PCR or sequencing reactions because of 3' dimerizing and hairpinning of oligonucleotide primers.	PCR, Sequencing	Select oligonucleotides with low dimer or hairpin formation potential at their 3' end.	*Duplex Formation Check* — Check the 3' ends of all oligos, using stability algorithms, eliminating those with dimer or hairpin potential.
Reduced efficiency PCR or sequencing reactions due to low general stability.	PCR, Sequencing	Select oligonucleotides with high or moderately high stability along their lengths, except for the 3' end.	*GC Clamp* — Determine stability (ΔG) of the 5' end and center segments of oligonucleotides; select only those with high or moderately high stability.
Very low efficiency PCR reactions because of 3' dimerizing between the upper (upstream) and lower (downstream) primers.	PCR	Select cross-compatible upper and lower primers (primers with low 3' dimer forming potential between them).	*Cross-compatibility Check* — Check the 3' ends of lower primers against the 3' ends of upper primers, using stability algorithms, eliminating those pairings with dimer potential.
Background due to false priming of the higher T_m primer in a PCR reaction.	PCR	Match the T_ms of the upper and lower primer.	*T_m Matching* — Add nucleotides to the 3' end of the lower T_m primer until its T_m most closely matches the higher T_m primer.
Reduced efficiency due to T_ms which are too low or too high for the application.	Sequencing, PCR, Hybridizations	Select oligonucleotides with optimal T_ms.	*T_m Window* — Select only oligonucleotides with T_ms within upper and lower T_m thresholds.
Ghost bands due to misalignment of primers or probes on the template.	Sequencing, PCR, Hybridizations	Select oligonucleotides that cannot misalign the template.	*Homooligomer and Sequence Repeat Checks* — Select only oligonucleotides which do not contain strings of the same nucleotide or sequence repeats.

Examples are continued on the following pages.

9. Set the ranges for a full search:
   a. Enter **350** to **600** for the positive strand primer search range and **800** to **1000** for the negative strand primer search range.
   b. Enter **400** to **600** for the PCR product length.
   c. Click "OK."
10. Click on the *Parameters* button.
11. Check that the *Search Stringency* is set to "high."
12. Make sure that *Adjust Length to Match T_m's* is marked.
13. Click "OK" to exit the "Parameters" window.
14. Click "OK" to start the search.
    The "Search Status" progress window appears, and the message "Done" appears when the search is complete.
15. Click on the "Primer Pairs" button.
16. Click on the circle above the "Sort by Product Length" column.
17. Use the scroll bar and/or arrows to find the desired product length closest to 500 (#8 is exactly 500 nt).
18. Click on #8 to select this pair and engage the "PCR Data" window.
19. From the *Window* menu, choose *Selected Primers* to review primer data for synthesis.
20. Review search results in the "Primers and Probes Search Data" window from the *Search* menu.

Example 4: Nested Primer Pairs

The OLIGO program permits automatic selection of two or more sets of cross-compatible (nested) primer pairs. Nested Primer selection requires a PCR primer search first (about 45 seconds on a 486/50 Mhz computer). To select a set of two primer pairs:

1. Choose *Reset* from the *File* menu and select "Data" to reset the parameters.
2. Choose the *Search for Primers and Probes* option from the *Search* menu, or press <F3>.
3. Make sure that all subsearch boxes are checked ***except*** "Continue False Priming Search in Other Files."
4. Activate the *Primer Pairs* function by clicking on the circle next to "Compatible Pairs."
5. Click on the "Search Ranges" button.
6. Enter **-18** to **150** for "Positive Strand Primer Search Range." Select **500** to **600** for "Negative Strand Primer Search Range." Enter **150** to **1868** for "PCR Product Length." Click "OK."
7. Click on the "Parameters" button on the "Search Parameters" dialog box and choose "Moderate" for the "Search Stringency."
8. Check to be sure the "Inverse PCR" box is not checked and that the "Match Tm's" box is checked. Click "OK" to return to the "Search for Primers and Probes" dialog box.
9. Click "OK" to start the search — 638 pairs will be accepted.
10. Click on the "Primer Pairs" button.
11. Choose *Multiplexing* from the *Analyze* menu.
12. Choose *Zoom In* from the control menu box (upper left corner of the window title bar) several times.
13. Select your outer pair of primers first by clicking directly on the square representing the primers. Select positions -9 and 593 for the first primer pair. The position numbers appear along the bottom of the screen.
14. Select the desired nested pair, positions 14 and 565. Note that this nested primer set is selected as close to the outside pair as possible without any primer overlap.
15. To move these primers to the database:
    a. Select *New Database* from the *File* menu, or press <Alt><N>.
    b. Click on the "Multiplexed Primers" button from the *Import Functions* menu at the bottom of the database screen. This will load the nested primers to the database.
16. You may want to examine database features in Example 11 at this point. To do this:
    a. Select *Analyze — Tm Graph* so you will be able to pick Upper and Lower Primers.
    b. Skip Step 2 in Example 11. You do not need to select *File — New Database*.

False Priming Analysis

Example 5: Single (Current) Oligo Analysis

The *Analyze* functions determine the technical and physical characteristics of a selected oligonucleotide or sequence.

In this demo, the *Analyze* function allows you to calculate:

- Duplex Formation
- Hairpin Formation
- False Priming Sites

To check the potential false priming sites of a single oligo on its template:

1. Choose *Reset* from the *File* menu and select "Data" to reset the Upper and Lower primers. Do not accept changes.
2. Verify that the oligo length is correct on the "Melting Temperature" window title bar. If it is not, change the length in the *Change* menu.

3. Select a current oligo of interest from the "Melting Temperature" window.
4. Select *Upper Primer* from the *Select* menu to select this "Current Oligo" as the upper primer. (Or, click on the "Upper" button at the left side of the T_m window, or press <Ctrl><U>.)
5. From the *Analyze* menu, choose the *False Priming Sites* function and *Upper Primer* option.

   The *Upper Primer False Priming Sites* information window appears. (Duplex and hairpin formations are performed in a similar manner as false priming analysis.)

PCR/LCR Analyses

In addition to single oligonucleotide analysis, OLIGO provides analysis functions for primer pairs, primer compatibility, PCR, and LCR. Primer pairs, primer compatibility, and PCR analyses are inactive until a search is completed.

Example 6: Creating a Restriction Site Map and Fragment Table for a DNA Template

1. With CBP.SEQ loaded, select the *Search For Restriction Sites* option from the *Search* menu to call up the "Search for Restriction Sites" window.
2. Click the "Entire" button to generate restriction data for the entire CBP file.
3. Click on the "Linear" button to get fragment sizes appropriate for a non-circular DNA sample.
4. Click both the "Map" and "Table" boxes to display search results in both formats.
5. Click "OK" to call up the "Select Enzyme File" window.
6. Click one of the three restriction enzyme files — non-degenerate 5/5+ cutters and 6/6+ cutters, plus the New England Biolabs Enymze list (Rebase.enz).
7. Click "OK" to start the search.

Example 7: Hybridization Time

Hybridization times can be calculated from the *Analyze* menu. To check an oligo's hybridization time:

1. Choose *Hybridization Time* from the Analyze menu.
2. For each of the following items — *Oligo Length, Concentration nM,* and *Concentration ng/ml* — click in the box and adjust the data to meet your conditions.
3. Press <Enter> to view new hybridization time calculations.

Example 8: Using the Concentrations Window

This example answers the question: "How many mL of buffer is required to produce a 10 µM solution with 2 OD units of the positive strand 21-mer (Current Oligo) at position 400?"

1. With an oligonucleotide length of 21, move the Current Oligo to position 400 using the *New Current Oligo Position* option from the *Select* menu, or press <F10>.
2. Select *Concentrations* from the *Analyze* menu.
3. Click the "Constant Concentrations" button to fix concentration while varying other parameters.
4. Click the "Current + Oligo" button.
5. Enter **2** in the [OD260] field and **10** in the µM field of the dialog box.
6. Click in any other field of the dialog box, or press <Enter> to reveal the answer (1.018 mL).

Example 9: LCR

The LCR function designs four oligos optimized for LCR to detect the presence or absence of a specific mutation. To select LCR primers designed to test for the presence of a mutation at position 50:

1. Choose *Reset* from the *File* menu and select "Data" to reset the Upper and Lower primers.
2. Click on the far left icon (circle with cross-hairs) at the bottom of the "Melting Temperature" window.
3. Enter **50** for the base number in the dialog box.
4. Choose *LCR* under the *Analyze* menu, or press <Ctrl><R>. (You can scroll through the sequence in this window using the mouse.)
5. Click on the "Select" button. The original and common oligos for both + and - strands appear.
6. Click on one or more of the remaining three circles to select the appropriate mutation. OLIGO displays the mutated oligo and the T_m recommended for the reaction.

Example 10: Reverse Translation

To use the reverse translation function (for short peptides):

1. Choose *Upper Primer* from the *Edit* menu.
2. Click on the protein sequence entry box just below the red border (the DNA box) to enable amino acid sequence entry.
3. Press several keys on the keyboard at random to enter a short amino acid sequence.

The keyboard input is scrambled in the demo version. You can delete directly in the sequence by highlighting parts of it and using the edit buttons. The yellow arrow just beneath the title bar is the "Undo" button.

4. Click on any residue in the sequence to view the human codon percentages for that residue.
5. To check codon usage in another organism, choose *Codon Table* from the *Change* menu.
6. Select a new organism by clicking on its corresponding file name. Click "OK."
7. To reverse translate the peptide using the codon table from this organism, choose *Entire Sequence* from the *Reverse Translation* menu.
8. Choose the appropriate option from the *Accept/Quit* menu. Most commonly, you will Accept and Quit, and analyze the new primer using the functions from the *Analyze* menu. However, you are limited to 100 nucleotides.

Example 11: Creating, Loading, and Using the Oligonucleotide Database and PRIMEFORM

The OLIGO program and the PRIMEFORM Oligonucleotide Ordering Software give you the ability to save your oligo sequences to a permanent database and send them to a synthesis ordering form. To review these features:

1. From the *Select* menu or using the "Upper" or "Lower" icons, choose an Upper and Lower primer, if not already selected. (Move the Current Oligo downstream from the Upper Primer to select a Lower Primer.)
2. From the *File* menu, choose *New Database*, or press <Alt><N>.
3. Once the database is loaded, under *Import Functions*, click on "Upper Primer" and then "Lower Primer," adding these primers to the database as records.

Note

Your primers have to be within previously established search ranges for the Priming Efficiency feature to work.

4. Examine the "Priming Efficiency" feature by clicking on the "All" button under the "Current" option and then the "PE" button.

This checks all the records (oligos) in the database against the active sequence file for priming efficiency. Two numbers are calculated — the priming efficiency of the most stable site on the active sequence file is listed first, followed by the highest theoretical PC (perfect homology hybridization). In this example, the numbers will match if the database oligos are from the active sequence file and within the set search ranges.

This feature may make it possible to reuse previously synthesized oligos on a new template. In order to have a reasonable chance of finding an oligo with a high enough PE (250+) to prime effectively on an average 2kb template, however, a 500+ record database of previously synthesized oligos to run against the template is recommended.

5. To send database primers to PRIMEFORM Oligonucleotide Ordering Software, click on the oligonucleotide record to export and then on the "Select/Deselect" button from the *Export Functions* menu. Do the same for additional primers. Notice that a dot appears in the first column, under the "O." This indicates that the record was sent to the order form.
6. Click on "Order Form" to call up PRIMEFORM and an order form containing the selected oligonucleotide sequences.
7. From the *Edit* menu, select *Accounting Information*.
8. Enter the information for your accounting department by clicking in the fields. To move from field to field, press <Tab> or use the mouse. When you are finished, click "OK" to return to the main menu.
9. From the *Edit* menu, select *Shipping Information*.
10. Enter the information by clicking in the fields. To move from field to field, press <Tab>. When you are finished, click OK to return to the main menu.
11. From the *Edit* menu, click on *Synthesis Specifications* to select the specific service, grade, scale/quantity and turnaround for your order.
12. To view your order form on screen, select *File — Print Preview*.

Note

You are unable to edit the header and footer information (*Edit — Order Information*) in this demo version.

PRIMEFORM

If you would like to use a standalone copy of PRIMEFORM to order oligouncleotide synthesis services from NBI, please:

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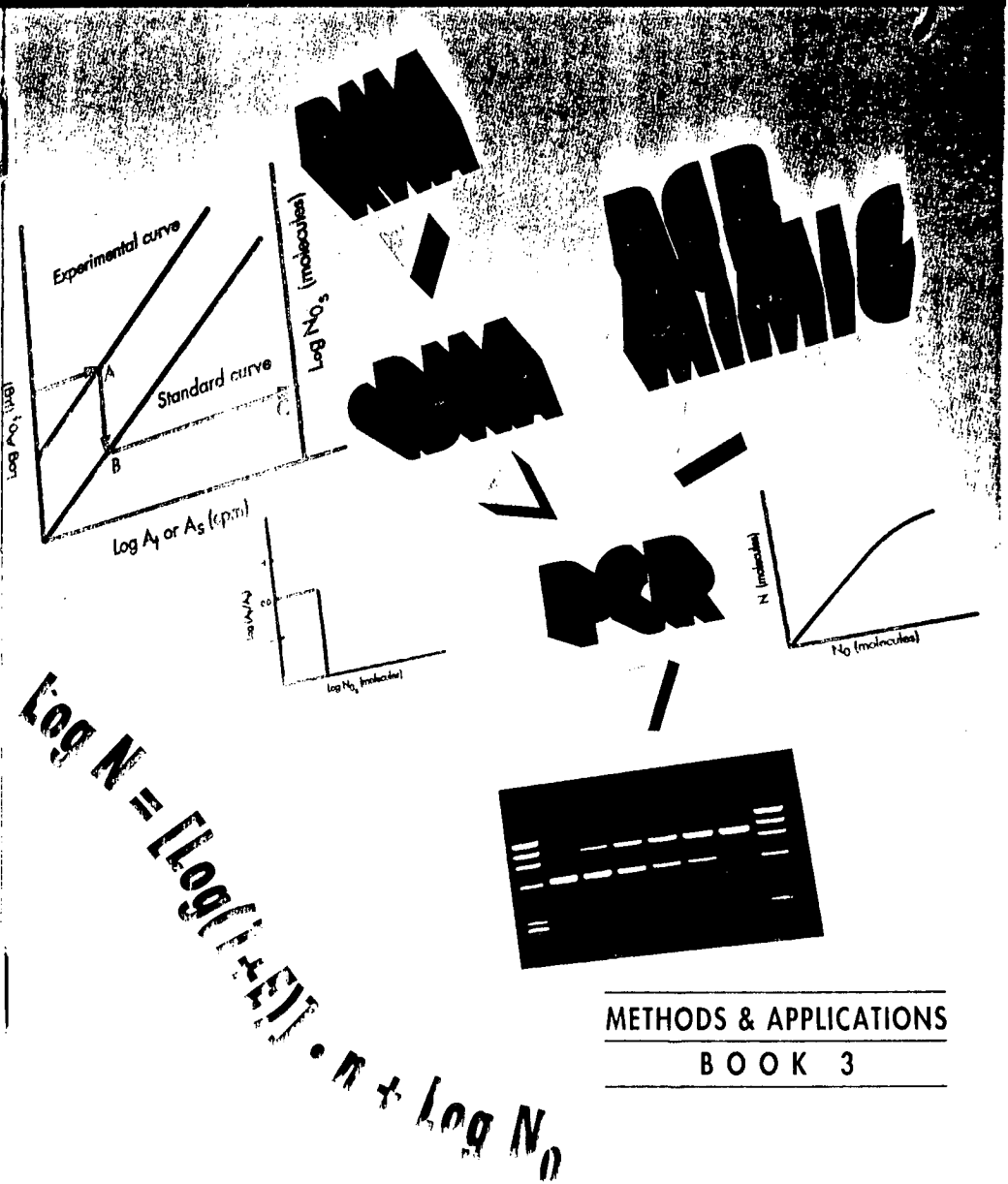
QUANTITATIVE RT-PCR



METHODS & APPLICATIONS

BOOK 3

CLONTECH Laboratories, Inc.

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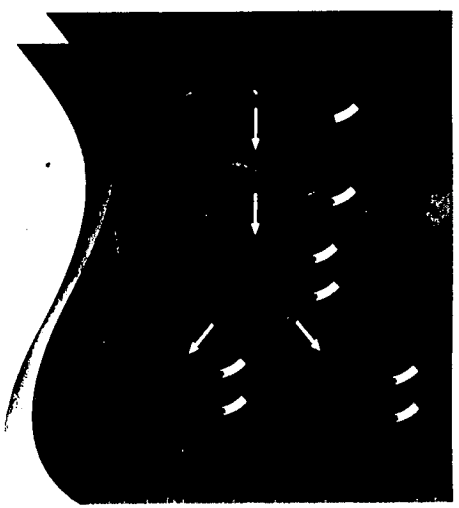
Murtaugh

RT-PCR



METHODS & APPLICATIONS

BOOK 1

CLONTECH Laboratories, Inc.

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FOREWORD

Reverse transcription of RNA followed by the Polymerase Chain Reaction (RT-PCR) is an extremely sensitive method to detect and quantify specific mRNAs. The purpose of this booklet is to provide an overview of the RT-PCR technique and its applications, supplemented with original data. We hope this will provide a basis for further investigation into specific areas of interest to the reader.

First Edition

Printed on recycled paper.
Printed with soy-based inks to reduce environmental impact.

Protocol Addendum

In this addendum we provide updated information on RNA isolation and an updated protocol for cDNA synthesis.

Notes and precautions:

- We recommend that you read through each protocol carefully before starting.
- When working with RNA and cDNA, always wear gloves to protect your samples from degradation by nucleases.
- To reduce the risk of DNA contamination of PCR products please read the DNA contamination section in Chapter 3.
- Always wear gloves when handling radioactive materials and dispose of radioactive waste properly.

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RNA Isolation

CLONTECH now has available the Micro-Scale Total RNA Separator Kit (#K1044-1), designed especially for RNA isolation for RT-PCR. The Micro-Scale Total RNA Separator Kit allows isolation of intact total RNA from very small amounts of cell or tissue samples. 15 mg of tissue or 1 x 10^6 cells can be used as starting material. The Micro-Scale Kit also comes with polyinosinic acid for use as a carrier nucleic acid so that RNA can be isolated from even smaller amounts of tissue or cells. The use of a carrier nucleic acid facilitates RNA handling and improves yields when working with very small samples. The use of both tRNA and polyinosinic acid has been documented, but we have had the most success with polyinosinic acid.

cDNA Synthesis

We typically synthesize cDNA from a maximum of 1 µg of total RNA in a total volume of 20 µl.

1. To 0.2–1 µg of RNA, add DEPC-treated water to give a volume of 12.5 µl. Incubate at 70–80°C for 3 minutes. Spin briefly and place on ice.

   Note: An optional step is to quantitate the yields of cDNA synthesis by monitoring incorporation of ^{32}P-dNTP by TCA precipitation. To do so add 5–10 µCi of ^{32}P-dNTP (e.g., dCTP) to the reaction. Adjust water in Step 1, so that the total volume is 20 µl. Remove a 1-µl aliquot prior to adding enzyme for the "minus enzyme" TCA precipitable cpm value. Then remove another 1 µl for determination of total ^{32}P-dNTP; dilute into 100 µl of water and count 1 µl of the dilution.

2. Add the following components:

Reagent	Amount	Final Conc.
20 µM oligo(dT)-18 primer or	1 µl	1.0 µM
20 µM random hexamer primers	1 µl	1.0 µM
5X reaction mix (Final concentration: 50 mM Tris-HCl, pH 8.3, 75 mM KCl and 3 mM $MgCl_2$)	4 µl	
dNTP mix, 10 mM each	1 µl	0.5 mM
Recombinant RNase inhibitor, 50U/µl	0.5 µl	1 U/µl
M-MLV reverse transcriptase, 200 U/µl	1 µl	1.0 U/µl
Total Volume	**20 µl**	

   (M-MLV: recombinant Moloney-Murine Leukemia Virus reverse transcriptase)

3. Incubate at 42°C for 1 hour. To quantitate yields by incorporation of ^{32}P-dNTP, remove 1 µl for the "plus enzyme" TCA precipitable cpm value.

4. Terminate reaction by incubation at 94°C for 2 minutes. Spin for 1 minute in a microcentrifuge at 4°C.

5. Dilute the cDNA synthesis reaction 5-fold by adding 80 µl DEPC-treated water. Vortex and spin again. The dilution will allow more accurate pipetting of the cDNA.

6. You will use 5–10 µl of the diluted cDNA in each 50-µl PCR reaction. The remaining cDNA can be stored at –70°C for several months or at –20°C for up to 1 week.

Table of Contents

(BKZ3003)

Introduction and Overview

Amplification of individual RNA molecules can be achieved by a method that combines reverse transcription and the polymerase chain reaction (PCR). Called RT-PCR here (1, 2), this method has also been termed RNA-PCR (3), RNA phenotyping (4), and message amplification phenotyping (5). RT-PCR has been shown to be thousands of times more sensitive than the traditional RNA blot techniques (6–7). This exquisite sensitivity gives RT-PCR the ability to detect extremely rare mRNAs, mRNAs in small numbers of cells or in small amounts of tissue, and mRNAs expressed in mixed-cell populations.

While RT-PCR is an extremely sensitive method of mRNA analysis, obtaining quantitative information with this technique can be difficult. This is due primarily to the fact that there are two sequential enzymatic steps involved: the synthesis of DNA from the RNA template and the polymerase chain reaction. In practice, the exponential nature of the polymerase chain reaction and the practical aspects of performing PCR pose the most serious obstacles to obtaining quantitative information. With some adaptations, however, RT-PCR can yield accurate quantitative results.

This booklet describes a number of methods that have been developed for using RT-PCR to determine the relative level of abundance of a particular mRNA, changes in the abundance of an mRNA over time or after induction, and the actual number of mRNA molecules in the sample. We will discuss the

theory and applications of each method, and the advantages and limitations associated with them. We will then expand on one method of quantitative PCR in particular, namely competitive PCR. We have found this method, using non-homologous internal standards (PCR MIMICs), to be both simple and useful.

We have tried to present a thorough discussion of the topic. However, due to the vast amount of available information about PCR and the rapid development of new methods, we may have inadvertently omitted some published information.

Theoretical and Practical Aspects of PCR

The Exponential Nature of PCR

By definition, the PCR process is a chain reaction. The products from one cycle of amplification serve as substrates for the next. Therefore, the amount of product increases exponentially and not linearly, as in most enzymatic processes. Under ideal or theoretical conditions, the amount of product doubles during each cycle of the PCR reaction according to Equation 1. This relationship is plotted in Figure 1A.

Equation 1: $N = N_0 2^n$

where:
N = The number of amplified molecules
N_0 = The initial number of molecules
n = The number of amplification cycles

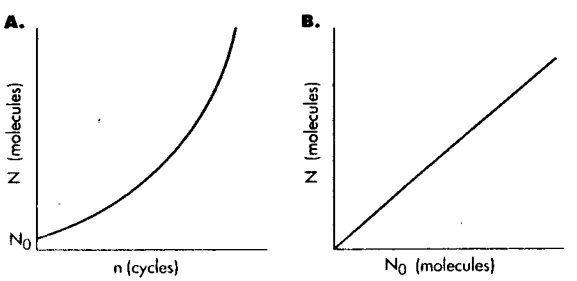

Figure 1. Characteristics of PCR amplification in an ideal case. Panel A: kinetics of amplification. Panel B: PCR product yield as a function of initial amount of target.

Equation 1 indicates a linear relationship between the number of amplified target molecules and the initial number of target molecules. This relationship is shown in Figure 1B.

The Efficiency of Amplification

Amplification efficiency, that is, the fraction of the template replicated during each reaction cycle, is a crucial factor for any reliable method of quantitative PCR. Experimentally, the efficiency of amplification (E) is less than perfect, and the PCR process is thus described by Equation 2.

Equation 2: $N = N_0 (1+E)^n$

where:
E = The amplification efficiency

Because of the exponential nature of PCR, a very small change in amplification efficiency, E, can yield dramatic differences in the amount of product, N, even if the initial number of target molecules, N_0, is the same. For example: if E = 0.85 and n = 30, then $N = N_0 (1+ 0.85)^{30}$ and $N = 10.4 \times 10^7 N_0$. In other words, PCR would produce a 10.4×10^7–fold increase in the amount of target molecules. However, if E is reduced to 0.8, the target would only be amplified 4.6×10^7 times by PCR. Thus, a change in amplification efficiency of only 0.05 would produce a greater than two-fold change in the amount of reaction product. This difference becomes even greater as the number of cycles increases.

Several experimental factors may affect the efficiency of amplification, including:

- The sequence being amplified
- The sequence of the primers
- The length of the sequence being amplified
- Impurities in the sample

The first three of these factors are important because they affect secondary structure formation and the G/C content of the target sequence—both of which may interfere with primer binding, affect the melting point of the target sequence, and reduce the processivity of the polymerase. The length of the target sequence being amplified can affect E for another reason: even with an ideal template, no polymerase exhibits 100% processivity under *in vitro* conditions. Because of the limited processivity of *Taq* DNA polymerase *in vitro*, target sequences longer than 3 kb are extremely difficult to amplify. More importantly, there is also some controversy about whether differences in target sequence lengths significantly alter the efficiency of amplification when the sequences are under 1 kb. In two cases, a weak inverse correlation was observed (8, 9). In another case, there was no observed difference in E (10). Impurities in the sample can affect amplification efficiency in many ways. For example, they can degrade or inhibit the polymerase, cause conformational changes in the target DNA, or compete for primer binding sites—to name just a few of the possibilities.

There may be additional, as yet unknown, often subtle, factors that affect E. This is illustrated by the fact that the amount of product amplified from the same target sequence after the same number of cycles and under identical experimental conditions often differs from one PCR reaction to another. This was seen even when using a master mix of reaction components (11, 12). Unfortunately, such tube-to-tube variation in amplification efficiency can be both significant and unpredictable. Theoretically, the efficiency of amplification, E, ranges from 0 to1. Experimentally, the value of E has been found to range from 0.46 to 0.99 for different genes (7, 10, 13). The value of E also varied, from 0.8 to 0.99, when the same gene was amplified in independent tubes under identical conditions (14).

The Plateau Effect

Experimentally, the amount of product generated during PCR also deviates from the theoretical case. The amount of PCR products produced during the polymerase chain reaction initially increases exponentially, but then the rate of production slows and finally levels off, as shown in Figures 2A and 2B. Figure 2A is a graph of the number of amplified target molecules (N) plotted as a function of PCR cycles (n), and Figure 2B is a graph of the number of amplified target molecules (N) plotted as a function of the initial number of target molecules (N_0). The leveling off of the rate of amplification is often referred to as the plateau effect.

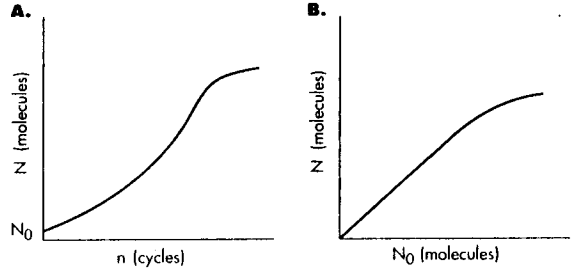

Figure 2. Characteristics of PCR amplification in a typical case. Panel A: kinetics of amplification. Panel B: PCR product yield as a function of initial amount of target.

The following factors may account for the observed plateau effect:

- The product accumulates to a concentration at which reassociation competes with primer annealing and extension (15).
- The molar ratio of polymerase to template falls below a critical value.
- Inhibitors of polymerase activity, such as pyrophosphates, may accumulate.
- One or more of the components necessary for the reaction become limiting.

The number of cycles needed to reach the plateau phase varies, depending on the sequence—and the original amount—of target mRNA. This variability makes it difficult to predict precisely the time course of the reaction or the amount of product synthesized before plateau phase is reached. The uncertainties inherent in the plateau effect, as well as the exponential nature of PCR, contribute to the difficulty of performing quantitative PCR because they obscure the linear relationship between N_0 and N depicted in Equations 1 and 2. Methods that employ RT-PCR to obtain quantitative information must take these factors into account.

Quantifying the PCR Product

The goal of quantitative PCR is to deduce, from the final amount of PCR product, either the initial number of target molecules (N_0) or the relative starting levels of target molecules among several samples. Thus, the first step in this process is to measure the amount of PCR product present.

Several methods are commonly used to quantify PCR products. The most straightforward approach is to measure the incorporation of labeled nucleotides or primers into PCR products resolved by gel electrophoresis. Although direct, the use of labeled nucleotides in PCR can be problematic. High levels of unincorporated, labeled nucleotides in the PCR product mixtures result from the relatively high (≥100 µM) concentrations of nucleotides required for PCR. Consequently, trace amounts of unincorporated label often remain in the electrophoretic gel as the product bands migrate, resulting in a "trail" of label throughout the lane. Even a relatively small amount of "trailing" can make it difficult to measure the amount of incorporated label. For this reason, many researchers prefer to use labeled PCR primers rather than labeled nucleotides.

Other strategies for quantifying PCR products are based on hybridization. The most common of these methods is to probe a Southern blot of the PCR products using a radioactively labeled probe designed to

hybridize to specific, amplified sequences. To quantitate the amount of probe hybridized, the blot can either be exposed to x-ray film and the resulting autoradiogram densitometrically scanned, or the PCR product band can be excised from the blot and its radioactivity measured in a scintillation counter. Because the nucleic acid probes only hybridize to the corresponding amplified DNA sequences, this method offers the advantage of detecting only the correct PCR product. Nonspecific products do not produce a signal.

Alternative hybridization methods that avoid Southern blotting have also been utilized (12, 16, 17). Jalava *et al.* (16) described an approach based on the capture and hybridization of biotinylated PCR products on streptavidin-coated microtiter plates. The biotin group is added to the PCR product during amplification through the use of a biotinylated primer. Biotinylated products are subsequently captured on streptavidin-coated plates, and a radioactively labeled nucleic acid probe, designed to hybridize to the biotinylated strand, is then used to measure the amount of captured product. Jalava *et al.* used relatively long, nick-translated DNA fragments (0.35 kb and 0.42 kb) as the radioactive hybridization probes; however, the results of their experiments suggest that it might also be possible to use short, nonisotopically labeled synthetic DNA probes, in conjunction with an appropriate detection system.

Another hybridization method that avoids Southern blotting is solution hybridization between a radioactively labeled probe and denatured PCR products. The hybridized probes are resolved by gel electrophoresis and subsequently quantitated by scintillation counting (12). Fluorescent labels also can be used instead of radioactivity. In this case, a fluorescently labeled internal primer is annealed to one strand of the PCR product and extended using *Taq* DNA polymerase. Run-off extension products are electrophoresed in an automated DNA sequencer that quantitatively detects the incorporated fluorescent label (17).

Several additional methods exist for quantifying PCR products. They include measurement of the EtBr luminescence emanating from PCR products resolved by gel electrophoresis (18), use of high performance liquid chromatography (19), and assays based on *in vitro* transcription with radioactively labeled ribonucleotide substrates (20). For *in vitro* transcription, a transcriptional promoter is incorporated into one of the PCR primers. Following amplification, the PCR product is transcribed *in vitro* using radioactively labeled ribonucleotides. During transcription, the radioactive signal is amplified 100- to 200-fold, making this a very sensitive detection method. However, the additional enzymatic reaction required for *in vitro* transcription makes this one of the more laborious detection methods and may also increase the risk of experimental error.

Chapter 2

Quantitative PCR without the Use of Internal Standards

Most commonly, researchers use internal standards to control variations in amplification efficiency and to determine absolute values of mRNA (discussed in the next section). However, it is possible to perform quantitative PCR without the use of internal standards if two conditions are met. First, tube-to-tube variation in the actual value of E must be minimal so that a constant value can be assumed for E in all related PCR reactions. Second, all data must be obtained before the reactions begin to reach the plateau phase. The methods described in this section employ mathematical models based on Equations 2 and 3 to determine relative changes in mRNA levels. At the end of this section is a discussion of the use of linear regression analysis (also based on these equations) to estimate absolute numbers of mRNA target molecules per unit of starting RNA without using internal controls.

Equation 2: $N = N_0 (1 + E)^n$

Equation 3: $\text{Log } N = [\text{Log } (1 + E)] \cdot n + \text{Log } N_0$

where:
N= The number of amplified molecules
N_0= The initial number of molecules
n= The number of amplification cycles
E= The amplification efficiency

For convenience, Equations 2 and 3 may also be written as:

Equation 2.1: $A = A_0 (1 + E)^n$

Equation 3.1: $\text{Log } A = [\text{Log } (1 + E)] \cdot n + \text{Log } A_0$

where:
A = The amount of amplified product (in cpm or OD_{260} units)
A_0 = The starting amount of total RNA (µg) or cDNA (ng). Note: the target sequences usually comprise only a small fraction of the total.

If the two conditions stated above are in effect (i.e., E is constant and reactions are not reaching the plateau phase), Equation 3.1 indicates that there is a linear relationship between the logarithm of the starting amount of target mRNA (or cDNA) (included in A_0) and the logarithm of the amount of amplification product generated (A). This relationship is illustrated in the graph of Figure 3. A linear relationship between Log A and Log A_0 has also been shown to exist empirically for values of A ranging over 2–3 orders of magnitude (21–23). In one case, this relationship was even found to hold for values of A differing by four orders of magnitude (18).

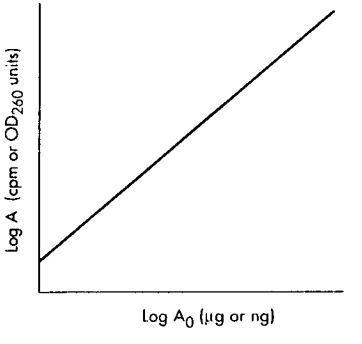

Figure 3. Linear relationship between the Log of the amount of PCR product and the Log of the initial amount of sample RNA (µg) or cDNA (ng), in an ideal case.

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Determining Relative Differences in N_0 between Two or More Samples

Two forms of experimental analysis, titration and kinetics, can be used to estimate the relative initial amounts of target mRNA or cDNA in two samples—when the amplification efficiencies are the same for the two samples and the data are collected before the reactions begin to reach the plateau phase.

Titration Analysis

A titration analysis is performed by making a dilution series, or titration, of RNA or cDNA, amplifying by PCR, and quantifying the signals produced (defined as A). Figure 4A shows idealized data collected from this type of experiment, graphed as Log A as a function of Log A_0. Because of the linear relationship between Log A_0 and Log A, and because the amount of target mRNA or cDNA is a constant proportion of the total starting material (A_0) for each of the various dilutions of a given sample, the relative difference in N_0 is proportional to the difference betwen the slopes of the two curves. Thus, a value of Log A_0 is chosen on the X (horizonal) axis of the graph and the corresponding values of Log A are then extrapolated for both curves, as shown in Figure 4A. The difference between the two values of Log A determined in this manner from the graph is equivalent to the relative difference in N_0 for the two samples. Singer-Sam *et al.* (23) used this method to determine the relative changes in mRNA levels for several phosphoglycerate kinases and phosphoribosyl-transferases during mouse spermatogenesis.

Kinetic Analysis

A more commonly used alternative to titration analysis is comparative kinetic analysis. To perform a kinetic analysis, values of A are determined for a number of consecutive amplification cycles (n) for two samples. Figure 4B shows idealized data from an experiment of this type, plotted as Log A vs. n. The curves are consistent with Equation 3. To determine the relative difference in N_0 between the two samples, a value of n is chosen at a point where the two curves are parallel (suggesting equal values for E), and the value of Log A is extrapolated from this value of n for each curve. At this point, the difference between the two values for Log A is directly proportional to the difference of Log A_0 between the two samples. Moreover, the difference of Log A_0 between the two samples is equal to the difference of Log N_0 between the two samples. Hence, this method can be used to determine **the difference** in the initial number of target molecules, but not the actual number of starting target molecules.

Equation 3: $\text{Log } N = [\text{Log } (1 + E)] \cdot n + \text{Log } N_0$

Comparative kinetic analyses have been used to accurately detect 2-fold (24) to 10-fold (26) changes in mRNA levels. For example, Solomon *et al.* (24) used this approach to examine differences in the levels of apolipoprotein mRNA in normal and atherosclerotic blood vessels. Dallman *et al.* (25) used a similar strategy to examine the influence of tissue transplantation on cytokine mRNA levels.

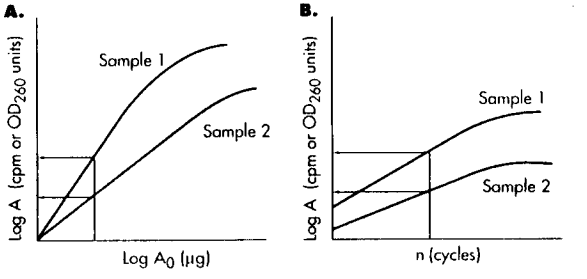

Figure 4. Two methods for determining relative differences in the initial amount of target in two samples.
Panel A: titration method. Panel B: kinetic method.

Using Linear Regression Analysis to Determine the Absolute Value of N_0

Equation 3 describes a linear relationship in the format, y = mx + b, whose slope (m) has the value of Log (1 + E) and whose y-intercept (b) is N_0. This allows estimation of the value of N_0 graphically. When the value of E is known, the value of N_0 can be determined from a linear regression analysis of the plotted data. Experimentally, a kinetic study is performed in which a constant amount of starting cDNA is amplified by PCR. During consecutive cycles, the number of product molecules, N, is determined. In this method it is necessary to calculate N, and not simply A. With the data graphed as Log N vs. n, E can be calculated from the logarithm of the slope, and N_0 can be derived from the y-intercept (Figure 5).

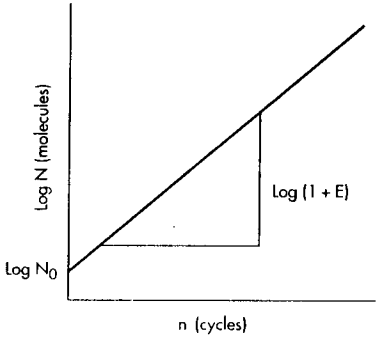

Figure 5. Determination of initial amount of target (N_0) and efficiency of amplification (E) using linear regression. Note that the slope = Log (1 + E) and the y-intercept = N_0.

This method was recently used by Wiesner (14) to estimate the number of α- and β-myosin heavy-chain mRNA molecules per unit of total RNA extracted from rat ventricle tissue. The authors also were able to calculate the number of mRNA molecules per cell, taking into account the yield of RNA and the number of myocytes per gram of tissue.

Quantitative PCR Using Internal Standards

Thus far, we have discussed a variety of methods for using quantitative PCR to determine relative initial levels of target mRNAs, and one method for estimating the absolute starting number of target molecules. However, in all of these methods, undefined variations in amplification efficiency (E) complicate the interpretation of results. In an attempt to correct for tube-to-tube variations in amplification efficiency, most investigators use internal amplification standards. Two types of internal standards can be used: an endogenous sequence or gene transcript that is normally present in the sample, or an exogenous fragment added to the amplification reaction.

Amplification of an Endogenous Sequence as an Internal Standard

An endogenous sequence, known to be present at constant levels throughout a series of samples to be compared, can be used as an internal standard in quantitative PCR reactions. Endogenous mRNA standards, typically for housekeeping genes or genes that are structurally or functionally related to the target mRNA (31), have been used to determine relative levels of specific mRNAs (20, 26–28). Furthermore, endogenous single-copy gene sequences have been used as internal standards to determine relative gene copy numbers (12, 29, 30). Finally, we know of at least one case where ribosomal RNA was used as an endogenous internal standard for quantitation of mRNAs (32).

In this approach, the endogenous standard sequence is amplified using a second pair of gene-specific primers, either in two separate PCR reactions, or in the same reaction as the target sequence. The ratios of the amount of PCR products generated by target and endogenous standard sequences in the different samples are then determined and compared. As with the methods described previously, the data from this type of experiment must be obtained before the amplification reactions reach the plateau phase. The data can be collected either from a titration of the sequences to be amplified, or by kinetic analysis, to ensure that signals are derived only from the exponential phase of the amplification.

The relative initial amounts of a target sequence and the endogenous standard (i.e., the ratio N_{0_t}/N_{0_s}) can be determined from Equation 4 (derived from Equation 2). (The subscripts "t" and "s" refer to the target and standard sequences, respectively.) Values for the efficiency of amplification (E) for the target and standard may be calculated from the slope of a graph of Log N as a function of cycle number (n). (See section on linear regression analysis, pg. 14). Note that when the amplification efficiencies of the two reactions—target and standard—are identical, i.e., $E_t = E_s$, the analysis is greatly simplified (10).

Equation 4:

$$N_{0_t} / N_{0_s} = N_t(1 + E_s)^n / N_s(1 + E_t)^n$$

where:

N_{0_t} = The initial number of target molecules
N_{0_s} = The initial number of standard molecules
N_t = The number of amplified target molecules
N_s = The number of amplified standard molecules
E_t = Amplification efficiency of the target
E_s = Amplification efficiency of the standard
n = The number of amplification cycles

However, even without a full mathematical analysis—and even in cases where E_s does not equal E_t—it has been shown empirically that endogenous mRNAs can be used to normalize target mRNA levels between samples to be compared. Thus, instead of determining the ratio of the intial absolute

amounts of target and standard using linear regression, the relative amounts of PCR products generated by the target and standard templates in different samples is simply compared. Although it has not been shown theoretically, Horikoshi *et al.* (20) suggested that if the internal standard mRNA is expressed at the same level in two samples, the ratio of PCR products generated from the target and standard should indicate the relative level of expression of the target mRNA in those samples. Furthermore, it may be true that if the target and standard are amplified in the same tube, tube-to-tube variations in amplification efficiency (due, for example, to pipetting error, sample impurities, or partially degraded RNA) may be minimized as well.

This type of approach has been experimentally validated by performing PCR on mixtures of DNA. For example, Horikoshi *et al.* (20) mixed specific ratios of DNA preparations from two cell lines, one with a documented 18-fold amplification of the dihydrofolate reductase (DHFR) gene and the other carrying the gene as a single copy. In this case, two independent PCR amplifications were performed on each sample using DHFR and β-actin primers, respectively, in separate reactions; the β-actin sequence served as a single-copy standard. Amplified products were obtained under conditions in which the amount of product was still increasing linearly with increasing amounts of starting sample (A_0). The ratio of DHFR to β-actin PCR products obtained from the mixtures differed by only ~30% from the predicted theoretical values.

In the experiment described above, the amplification of standard and target sequences was conducted in separate PCR reactions. However, a close correlation between predicted and observed target levels was similarly found by Neubauer *et al.* (29), who performed both amplifications in a single PCR reaction in a method they referred to as differential PCR. In this case, the authors were investigating the loss of the β-interferon gene in chronic myelogeneous leukemia; the target was the β-interferon gene and the standard was the γ-interferon gene. They were able to detect changes as small as 2:1 and 3:2 in the

ratio of the two genes using this method. Coamplification also was used by Chamberlain *et al.* (30) to examine exon deletions in the Duchenne muscular dystrophy locus. In an approach they called multiplex DNA amplification, they simultaneously amplified (in one tube) six exons, each with a different set of primers. In another example, Kellogg *et al.* (12) corrected for the effects of variable amplification efficiency of an HIV-1 DNA template in several samples by using a single-copy gene from the HLA locus as a reference standard.

Many examples of the use of endogenous mRNA standards to determine relative levels of specific mRNAs (in the same tissue) can be found in the literature. The first group to use this approach was Chelly *et al.* (10), in a study of dystrophin gene expression in different muscle tissues. Chelly *et al.* used aldolase A mRNA as the internal standard, and they performed the mathematical analysis, including calculation of amplification efficiencies, described at the beginning of this section. Noonan *et al.* (26) studied the relative expression of the multiple drug resistance gene (*mdr*-1) in tumor cells by normalizing PCR data to β_2-microglobulin mRNA. Horikoshi *et al.* (20) investigated expression of thymidylate synthase mRNA in tumor samples using both β_2-microglobulin and β-actin mRNA as endogenous standards. Murphy *et al.* (27) utilized both target titration and kinetic strategies to examine *mdr*-1 mRNA levels in tumor cells. Finally, Kinoshita *et al.* (28) examined levels of T-cell leukemia virus type I by performing a detailed kinetic PCR analysis that used β-actin mRNA as the endogenous standard.

Perhaps the greatest advantage of using the expression of an endogenous sequence as an internal standard is that the reference mRNA and the target mRNA are usually processed together for the entire duration of the experiment—from RNA extraction through PCR amplification. This tends to minimize differences in RNA yield between samples—an important advantage, particularly for analysis of small tissue samples where the quantities of RNA are too small to measure by UV spectrophotometry. In addition, if the entire population of mRNA is converted to cDNA by the use of oligo(dT) primers or random hexamers, the overall efficiency of cDNA synthesis also is somewhat normalized.

Notwithstanding the advantages to this approach, several complications may arise when amplification of endogenous mRNAs is used for semi-quantitative analysis. For this method to be reliable, the level of expression of the reference standard must be the same in each sample to be compared and must not change as a result of the experimental treatment. Unfortunately, few if any genes are expressed in a strictly constitutive manner. This is even the case for many housekeeping genes, including β-actin (33, 34). Therefore, the level of the mRNA used as the endogenous standard must be examined very carefully to ensure its constancy among all of the experimental conditions studied.

Another challenge of this approach is to obtain values of A_t and A_s before the amplification reactions reach the plateau phase, especially when the relative levels of expression of the standard and target sequences differ greatly. For example, if β-actin mRNA is used as the internal standard, it may be present at a much higher level than the target transcript, and amplification of the control may approach plateau phase well in advance of that of the target sequence. Indeed, Murphy *et al.* (27) found that the internal standard mRNA, for β_2-microglobulin, entered the plateau phase before the target, *mdr*-1 mRNA, was even detectable. One clever solution to this problem involves simply waiting until later stages of the amplification before adding the primers for the endogenous standard (28). Other researchers used gene-specific primers to synthesize cDNA from the control and target mRNAs in separate tubes and then mixed dilutions of the control and target cDNAs before performing multiplex PCR (32).

Interference is a frequently observed problem when more than one set of primers is used in the same PCR reaction. For example, when Murphy *et al.* (27) added both β_2-microglobulin and *mdr*-1 primers to the same PCR reaction, they observed a premature attenuation of the exponential phase of both PCR

amplifications. At CLONTECH we have observed similar results; the amount of product generated (from either the target, the standard, or both) is often dramatically reduced when both sequences are amplified in a single reaction. In fact, primer pairs that function truly independently seem to be the exception rather than the rule.

Amplification of an Exogenous Sequence as an Internal Standard

Exogenous sequences can also be used as internal PCR standards. In this approach, an exogenous mRNA or DNA standard is added to the target sample and amplified simultaneously with the target transcript in a single PCR reaction mixture. The exogenous standard can be either a synthetic RNA added to the reverse transcription reaction or a DNA, not normally in the target sample, that is added directly to the PCR reaction.

The theory behind use of exogenously added gene sequences as internal standards is similar to that described above for endogenous reference sequences. With both types of internal controls, the amount of amplified standard can be quantified after the experiment, and the change in the amount of standard is proportional to the change in the amount of target. However, there is a significant advantage in using an exogenously added sequence as the internal control; namely, the initial amount of standard used in the PCR reaction is precisely known. This makes it possible to calculate the absolute level of target mRNA or cDNA present in the original sample.

A common method of obtaining quantitative results from PCR with an exogenous standard involves generating a standard curve from the data collected. This method was first described by Wang *et al.* (7), who quantified changes in the levels of several cytokine mRNAs in stimulated macrophage cells using a synthetic internal RNA standard. In this approach, the RNA standard shares primer binding sites with the target RNA, but posseses a different "stuffer" sequence and an oligo(dT) tail. A known

amount of the RNA standard is mixed with a known quantity of RNA sample (measured in micrograms, for instance) and reverse transcribed. A series of PCR reactions is then set up with dilutions of the cDNA. Because the titration is performed on a defined mixture of the target and standard mRNAs, and because the mixtures are not titrated against one another, this is not a competitive reaction (competitive PCR is discussed later). This strategy simply allows the generation of two titration curves: one for the standard RNA and one for the target RNA.

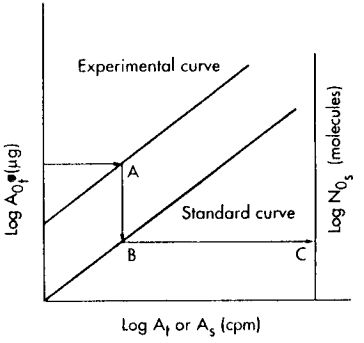

Figure 6. Use of a standard curve, derived from an exogenously added internal standard, to quantitate initial amount of target.

Following PCR, the amounts of PCR products obtained from the standard (A_s) and target (A_t) sequences are determined, and two curves are plotted, as illustrated in Figure 6. The RNA standard curve is generated by plotting the logarithm of the starting number of RNA standard molecules (Log N_{0_s}) on the right vertical axis as a function of the logarithm of the amount of amplified standard product (Log A_s). The target RNA curve is generated by plotting the logarithm of the initial amount of RNA sample (Log A_{0_t}) on the left vertical axis as a function of the logarithm of the amount of target amplification products (Log A_t). To determine the number of target mRNA molecules per unit of total RNA, a value of A_{0_t} is chosen in the region where the curves

are parallel (e.g., where values for E are identical). A line is drawn from that point (labeled A in Figure 6) down to the internal standard curve (point B), and from point B, a line is drawn across to the right vertical axis (point C). The value at this point is taken as the starting number of target molecules, N_{0_t}, in each microgram of total RNA. If the amount of total RNA per cell is known, the actual number of target mRNAs per cell can also be calculated. In the study by Wang *et al.* (7), changes in mRNA levels of 3-fold or less were reproducibly discernible. Also, the results correlated well with data obtained from a Northern blot analysis. A similarly close correlation between this method of quantitative PCR and Northern blot analysis was found by Prendergast *et al.* (35).

A critically important requirement of this type of experiment is that the value of E be the same for both the target and standard mRNAs. This can be accomplished by designing the standard to contain the same primer binding sequences as the corresponding target mRNA. In many cases this is sufficient to make E_s equal to E_t. Additional requirements for using exogenous standards are that the PCR products be of similar size and under 1 kb. At CLONTECH we have observed, as did Wang *et al.* (7), that the primer sequences have the greatest effect on amplification efficiency when the sizes of the amplified sequences are similar. Wang *et al.* showed that the amplification efficiency of an RNA standard was the same as that of its corresponding target even though the sequence between the shared primer binding sites was completely different. Of course it is important that no regions of significant secondary structure differ between the target and standard RNA sequences. Differences in efficiency still may exist, so this parameter should always be examined before drawing firm conclusions from each study.

To calculate the absolute initial number of target molecules (A_{0_t}), the initial number of standard molecules (A_{0_s}) must be known, and a method to differentiate between the number of amplified standard and target molecules (A_s and A_t, respectively) must be available. The most common technique used to distinguish between A_s and A_t is to make their sizes sufficiently different such that they can be resolved by polyacrylamide or agarose gel electrophoresis. Probe hybridization also can be used if the sequence between the two primer binding sites differs. In some cases, different restriction sites within the sequences between the primer binding sites can be used to differentiate target from standard—simply by digestion with an appropriate restriction endonuclease prior to gel electrophoresis.

Since the study by Wang *et al.* was published, several reports have described the construction of exogenous RNA and DNA internal standards that differ from target sequences only by the presence or absence of small introns or restriction sites (36–38). In these cases, there is little doubt that the amplification efficiencies of the standard and target sequences will be the same.

Competitive PCR

Competitive PCR also uses an exogenous template as an internal standard. However, the amplification takes place in a truly competitive fashion because the standard and target sequences actually compete for the same primers and, therefore, for amplification. In competitive PCR, a dilution series is made of either the target sequence or the standard sequence, and a constant amount of the other component is added to each of the reactions. Quantification is performed after competitive amplification of the entire series of reactions and is achieved by distinguishing the two PCR products from each tube by differences in size, hybridization properties, or restriction enzyme sites. An important advantage of competitive PCR is that, because the ratio of target to standard remains constant during the amplification, it is not necessary to obtain data before the reaction reaches the plateau phase.

In competitive PCR, the competitor fragment (usually DNA) takes the place of the standard described in the experiments discussed in the previous sections. We will continue to call it the standard, and use the symbol 's' to designate it in equations. When the amplification efficiencies of the target and standard molecules are the same, Equation 4 can be simplified to Equation 5.

Equation 5: $N_{0t} / N_{0s} = N_t / N_s = A_t / A_s$

where:
N_{0t} = The initial number of target molecules

N_{0s} = The initial number of standard molecules
N_t = The number of amplified target molecules
N_s = The number of amplified standard molecules
A_t = The amount of amplified target (in cpm or OD_{260} units)
A_s = The amount of amplified standard (in cpm or OD_{260} units)

Thus, for any value of n, the initial ratio of target to standard is equal to the ratio of their amplification products (i.e., N_t/N_s or A_t/A_s). This has been demonstrated both theoretically (39) and empirically (40). Therefore, if the standard and target sequences amplify with the same efficiency, the absolute initial amount of target cDNA (and in turn target mRNA), can be determined by allowing known amounts of standard (DNA) molecules to compete with the target for primer binding during amplification.

In the competitive PCR method illustrated in Figure 7, a dilution series of the DNA standard (referred to in the figure as the "MIMIC"*) is made, and these dilutions are added to a series of PCR reactions containing a constant amount of sample cDNA. Following PCR, the amplification products are analyzed by gel electrophoresis, and the amount of products generated by the standard (A_s) and the target (A_t) are determined for each individual reaction. The logarithm of the ratio of A_t/A_s is graphed as a function of the logarithm of the initial molar amount of the standard (N_{0s}) (Figure 8). The initial amount of target cDNA (N_{0t}) in the reaction is extrapolated from the graph, assuming that N_{0t} is equal to the amount of the standard (N_{0s}) added, in the reaction that produces an equimolar ratio of the two types of products (i.e., where the Log of A_t/A_s = Log of 1/1 = 0). Note that if there is a difference in the size of the standard and the target sequence, N_{0t} does not precisely equal N_{0s} (because longer sequences incorporate more label than shorter ones). Thus, a corresponding correction must be made in the calculation of N_{0t}.

*The use of PCR MIMICs is discussed on page 32.

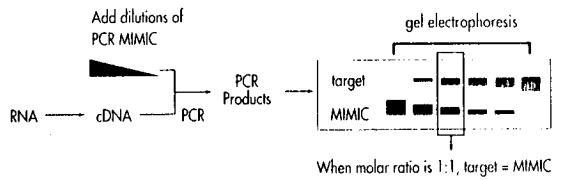

Figure 7. Schematic diagram of competitive PCR utilizing a competitor DNA fragment (PCR MIMIC), differing in size from the target sequence. A dilution series of the competitor is added to a constant amount of cDNA. Following amplification, samples of the PCR products are resolved by gel electrophoresis, and the yields of amplified competitor and target products are quantified. The relative amounts of target product and MIMIC product in each sample are compared. The initial amounts of target and competitor are assumed to be equal in those reactions where the molar ratio of target and competitor products are judged to be equal (after correction for size differences). Because the amount of competitor added to each PCR reaction is known, the absolute initial amount of target can be determined. If the competitor is a synthetic RNA, a dilution series of the competitor is added to a constant amount of sample RNA before the reverse transcription step.

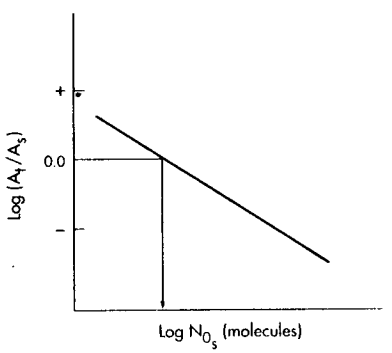

Figure 8. Analysis of the results of a competitive PCR experiment, such as that illustrated in Figure 7. The Log of the ratio of amplified target to competitor products is graphed as a function of the Log of the known amount of competitor added to the PCR reaction. Note that when the molar ratio of target and competitor is equal to 1, the Log of that ratio is equal to 0.

In general, when determining absolute initial amounts of mRNAs by competitive PCR using standard DNA fragments, one must take into account the fact that the efficiency of reverse transcription is less than 100%. The efficiency of cDNA synthesis using oligo(dT) as a primer for cDNA synthesis has been reported to be 40–50% (40, 41). Thus, calculations such as those described above indicate the minimum number of mRNA molecules present in a given sample.

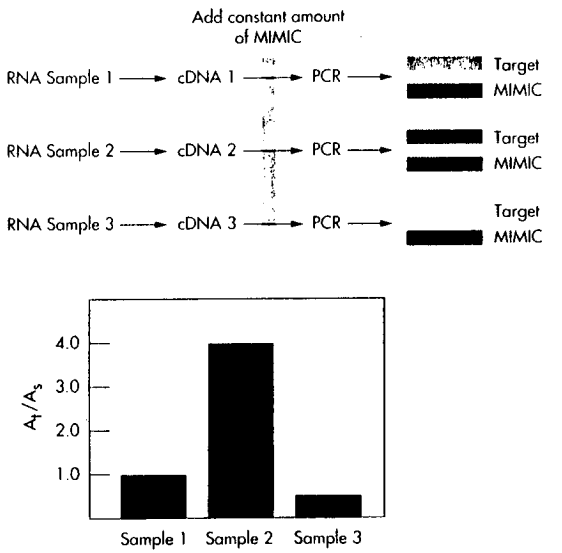

Figure 9. Semi-quantitative competitive PCR using a PCR MIMIC. A constant amount of competitor (PCR MIMIC) is added to PCR reactions containing the experimental cDNA samples. The ratio of amplified target to competitor PCR products is then determined for each sample. Differences in the ratios indicate the relative differences in mRNA levels between the samples.

As shown by Price *et al.* (42), it is not necessary to conduct a full dilution series of the competitor to determine relative changes in mRNA levels. Price *et al.* added a constant amount of competitor to PCR reactions containing the cDNA samples, as illustrated in Figure 9. They then estimated the relative

abundance of the target mRNA among the samples by comparing the ratio of the amount of amplified target to amplified standard in each sample. The increase or decrease in this ratio from sample to sample directly reflected the initial relative abundance of the target mRNA within each sample. This semi-quantitative approach is more convenient than using dilutions of the standard, especially when studying multiple samples.

Homologous Competitor Fragments

Becker-André and Hahlbrock (37) and Gilliand *et al.* (38) were the first to describe competitive PCR using homologous competitor fragments. Gilliand *et al.* used two types of internal standard: a genomic fragment corresponding to the target mRNA sequence but containing a small intron (thus yielding a PCR product slightly larger than the target mRNA); and a cDNA which was modified to contain a unique restriction site. In the latter case, PCR products were digested with the appropriate restriction enzyme before electrophoresis to differentiate between target and competitor products. To generate their internal standard, Becker-André and Hahlbrock used an *in vitro* transcribed mRNA designed to be identical to the target mRNA sequence except for the addition of a unique restriction site. They added different amounts of the competitor RNA into reverse transcription reactions containing a constant amount of target RNA.

Although it may be relatively easy to perform restriction endonuclease digestions to differentiate between target and competitor products, construction of such competitor fragments is often not a trivial matter. Unless there is a known small intron in the target gene, the construction of homologous competitors can require time-consuming site-directed mutagenesis and multiple cloning steps. However, recently several clever methods that use simple PCR amplification with composite primers (43–45) have been developed to generate homologous DNA standards. These methods can be extended to yield homologous competitor RNAs as well.

One potential problem with the use of competitor fragments that are homologous to the target is that during later stages of PCR, when the concentration of products is high, heteroduplexes can form between the standard and target sequences. This can complicate quantification of the PCR products derived specifically from the target or the standard, particularly when restriction enzyme digestion is required to distinguish between them. Therefore, heterologous DNA standards may be preferable (discussed in the next section).

While heteroduplex formation often interferes with obtaining accurate quantitative results from competitive PCR, in a novel approach described by Henco and Heibey (46) it is the heteroduplexes that are actually quantified. A known quantity of an internal standard, which is identical to the target except for a single nucleotide, is added to a dilution series of the target sample. Following PCR, a trace amount of radioactively labeled standard is added to the PCR products. The mixture is denatured and allowed to re-anneal; the labeled standard anneals to both target and standard sequences as a tracer. The homoduplexes and heteroduplexes are then resolved by temperature-gradient gel electrophoresis, and the amount of material in the heteroduplex (reflecting the amount of amplified target) is quantified.

Heterologous Competitor Fragments

DNA fragments that share the same primer template sequence but contain a completely different intervening sequence can also be used for competitive PCR. Überla (47) prepared fragments for competitive analysis by amplifying genomic DNA fragments from another species with a low annealing stringency. Siebert and Larrick (48) ligated the primer template sequences to a nonhomologous DNA fragment to generate DNA standards (competitor fragments). More simply, the competitive DNA standard can be obtained by amplification of a heterologous DNA fragment using composite primers. We used this last approach to construct competitive fragments (called PCR MIMICs™) at CLONTECH.

CLONTECH's PCR MIMIC™ Strategy for Competitive PCR

Background

Initially, we explored the possibility of using an endogenous mRNA as an internal standard to obtain quantitative data in experiments using our cytokine RT-PCR primers. We tried many different primer sequences, designed to amplify different endogenous mRNAs, but we repeatedly observed that almost every primer pair interfered with amplification of either the target or the standard (or both) when used in the same PCR reaction. We then examined the use of an exogenous standard having the same primer binding sequences as the target. We generated the standard by ligating the target primer sequences to a 600-bp heterologous DNA fragment of the viral oncogene v-*erb*B containing different restriction sites on either end. The standard amplified very efficiently and with similar kinetics to the target even though the sequences between their respective primer binding sites were completely different. Furthermore, we found that these exogenous sequences worked very well as internal standards in competitive PCR experiments (48). We refer to these heterologous standards as PCR MIMICs because they "mimic", or closely imitate, the primer binding and amplification characteristics of the target. We have since found an even simpler method for generating heterologous competitive PCR standards of virtually any desired size.

Generation of PCR MIMICs

PCR MIMICs are generated by two successive PCR amplifications as shown in Figure 10. In the first PCR reaction, a heterologous DNA fragment is amplified using two composite primers. One composite primer contains the upstream primer for the target sequence linked to a 20-mer that anneals to one strand of the heterologous DNA fragment. The other composite primer contains the downstream primer for the target sequence linked to a 20-mer that anneals to the opposite strand of the heterologous DNA fragment. The two composite primers are used to amplify a small fragment of the heterologous DNA. During amplification, the target-specific primer sequences are incorporated into the PCR product. This PCR product is diluted and used to perform a second PCR amplification with primers for the target gene only. In this way the entire target primer sequences are incorporated onto the ends of the heterologous DNA fragment.

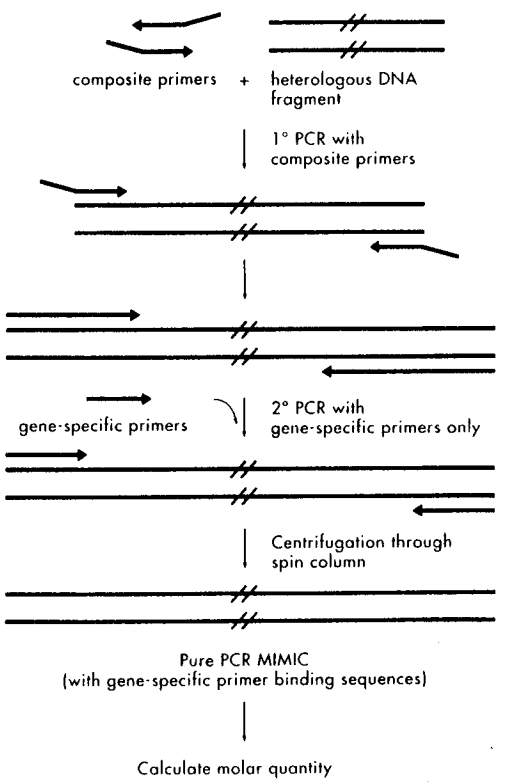

Figure 10. Flow chart illustrating the generation of competitive PCR MIMICs. Note that the composite primers are composed of two sections; the 3' portion anneals to the heterologous DNA fragment and the 5' portion anneals to the specific target gene.

The PCR product, the newly generated PCR MIMIC, is purified by passage through a spin column that removes PCR reaction components and primers. The quantity of PCR MIMIC obtained is then determined either by measuring the absorbance at 260 nm or by running an aliquot of the PCR MIMIC on a gel and comparing the intensity of the band to a dilution series of DNA markers containing known quantities of DNA. The mass quantity is then converted to molar quantity using the approximation that 1 nanogram of a 300-bp DNA fragment is equal to 5 x 10^3 attomoles (1 attomole = 10^{-18} moles).

A typical yield of PCR MIMIC, approximately 200 nanograms, is enough to perform hundreds of competitive PCR experiments. Therefore, in practice a single determination of MIMIC yield can be used for all experiments. In this way inaccuracies in yield determination will not affect the determination of relative changes in mRNA levels (from a series of samples) by competitive PCR.

PCR MIMICs are designed so that the size of the PCR product generated from them is either slightly larger or slightly smaller than the PCR product generated from the target sequence. MIMICs of different sizes can be made simply by designing the composite primers so they anneal to different regions on the heterologous DNA fragment.

Validation of the PCR MIMIC Strategy

The theory behind the use of PCR MIMICs (i.e., heterologous competitor fragments) in quantitative PCR has been discussed (pp. 24–27). There it was reasoned that if the competitor fragment and the target sequence amplify with the same efficiency, for any value of n, the initial ratio of target to standard (i.e., N_{0t}/N_{0s}) is equal to the ratio of their amplification products (A_t/A_s). Because PCR MIMICs share the same primer binding sequences as their corresponding target fragments, they are expected to have similar amplification efficiencies (ref. 7 and discussion on pg. 22). However, because the amplified target and MIMIC sequences flanked by the primer binding sites are different, it was important to verify that target/MIMIC pairs can amplify their respective sequences with similar efficiencies. To do this, we compared the amplification kinetics of several target/MIMIC pairs for a number of human gene transcripts, including those for the ubiquitously expressed housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and β-actin, as well as for the cytokines IL-1β, IL-4, and IFNγ.

The results of the comparative kinetic analyses of the amplification of the G3PDH MIMIC and target are shown in Figure 11. In this study, approximately equal molar quantities of a G3PDH target cDNA and the G3PDH MIMIC were added to a single PCR reaction along with a small amount of α^{32}P-dCTP. Aliquots were removed after each cycle for a total of seven cycles, starting when PCR products were first visualized on an agarose gel. The agarose/EtBr gel profile of the samples is shown in Figure 11A. The bands corresponding to the target and MIMIC were then excised from the gel and the amount of radioactivity measured in a scintillation counter. The logarithm of the amount of amplified target (Log A_t) and of amplified MIMIC (Log A_s) were graphed as a function of cycle number (n) (Figure 11B). The linear portion of the two curves had very similar slopes, indicating that the G3PDH target and MIMIC had very similar amplification efficiencies.

Similar results were obtained in the comparative kinetic studies of the other MIMIC/target pairs mentioned above (data not shown). In all cases, the target sequence and its corresponding MIMIC amplified with similar efficiencies, even though they possess different sequences between their respective primer binding sites. These findings are consistent with previous studies that have shown that the sequence of the primers has the greatest effect on amplification efficiency (7).

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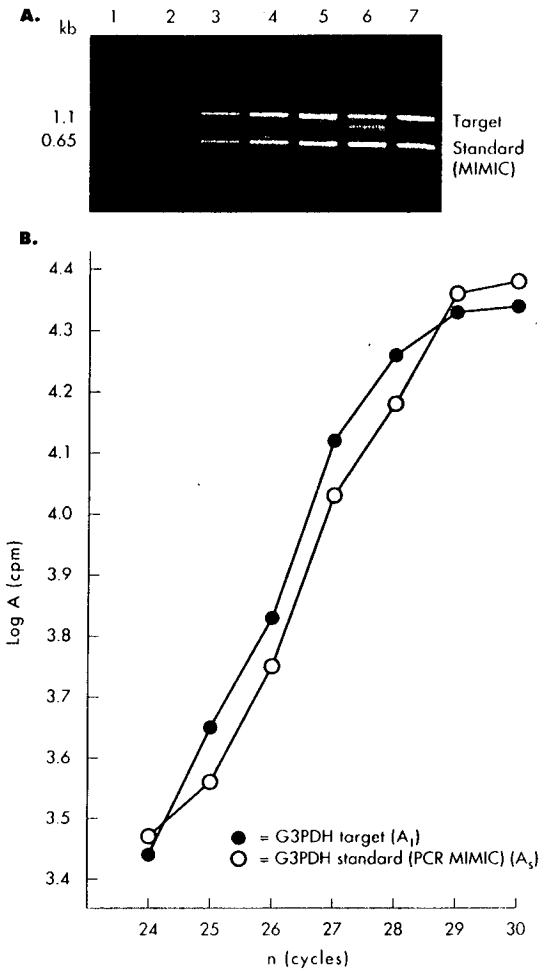

Figure 11. Kinetics of amplification of the G3PDH target cDNA and the G3PDH PCR MIMIC. 0.1 attomole (each) of the G3PDH target and MIMIC were added to a PCR reaction together with α^{32}P-dCTP. Panel A: After 24 amplification cycles, and after each of six additional cycles, a small portion of the reaction was removed and the products resolved on a 1.8% agarose/EtBr gel. Panel B: Following gel electrophoresis, the bands corresponding to the target (1.1 kb) and MIMIC (0.65 kb) were excised from the gel and the amount of radioactivity in each band determined by scintillation counting. The Log of the amount of PCR product (cpm) was graphed as a function of cycle number.

An example of a competitive PCR experiment using PCR MIMICs is shown in Figure 12. Ten-fold serial dilutions of the G3PDH MIMIC were coamplified with a constant amount of a cloned G3PDH cDNA fragment. Again the reactions contained a small amount of α^{32}P-dCTP. After amplification, a portion of each sample was resolved by gel electrophoresis (Figure 12A). The bands corresponding to the target and MIMIC amplification products were excised and the amount of radioactivity present in each of the excised bands determined by scintillation counting. The logarithm of the ratio of target to MIMIC amplification products (i.e., Log $[A_t/A_s]$) was graphed as a function of the logarithm of the amount of MIMIC DNA added (i.e., Log N_{0s}) (Figure 12B). After correction for the size difference between the MIMIC and the target PCR products, the equivalence point was deduced to be at 0.075 attomoles. This value was in very close agreement with the amount of target cDNA added to the PCR reaction (0.1 attomoles).

Next we examined the ability of competitive PCR to accurately measure a relatively small change in the level of a specific mRNA. For this purpose we prepared a PCR MIMIC for human IL-1β. To imitate a 4-fold induction of IL-1β mRNA, we synthesized cDNA from both 0.5 µg and 2 µg of total RNA. Competitive PCR was then performed with serial dilutions of the IL-1β MIMIC, and samples of the PCR products were electrophoresed on agarose/EtBr gels (Figure 13A). The sizes of the target and MIMIC PCR products were 0.80 and 0.65 kb, respectively. The bands corresponding to the IL-1β target and IL-1β MIMIC products were excised from the gel, and the amount of radioactivity measured by scintillation counting. The logarithm of the ratio of IL-1β target to IL-1β MIMIC amplification products (i.e., Log $[A_t/A_s]$) was graphed as a function of the logarithm of the initial molar amount of IL-1β MIMIC (i.e., Log N_{0s}) (Figure 13B).

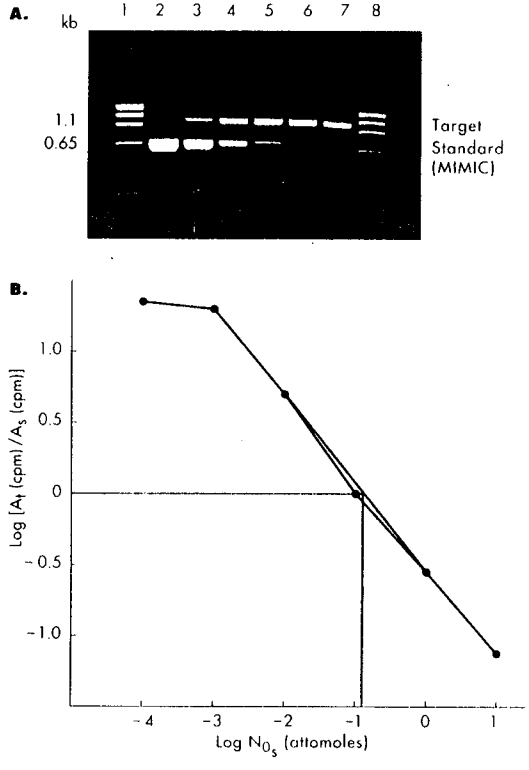

Figure 12. Validation of competitive PCR analysis using known quantities of a cloned gene fragment. 0.1 attomole of G3PDH cDNA was added to a PCR reaction containing 10-fold serial dilutions of a G3PDH MIMIC. α^{32}P-dCTP was included in the reaction to allow quantitation of the PCR products. After 30 amplification cycles, small portions of the products were resolved on a 1.8% agarose/EtBr gel. Panel A. Lanes 2–7: 10^1, 100, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} attomoles of G3PDH MIMIC, respectively. Lanes 1 & 8: ϕX174/*Hae* III DNA size markers. The positions of the 1.1-kb G3PDH target and the 0.65-kb G3PDH MIMIC PCR products are indicated. Panel B. Following electrophoresis, the bands corresponding to the target and MIMIC (shown in Panel A) were excised from the gel and the amount of radioactivity determined by scintillation counting. The relative amounts of target and MIMIC products were calculated after correcting for the difference in size between them. The Log of the ratios of target products to MIMIC products were graphed as a function of the initial amount of MIMIC added to the PCR reactions.

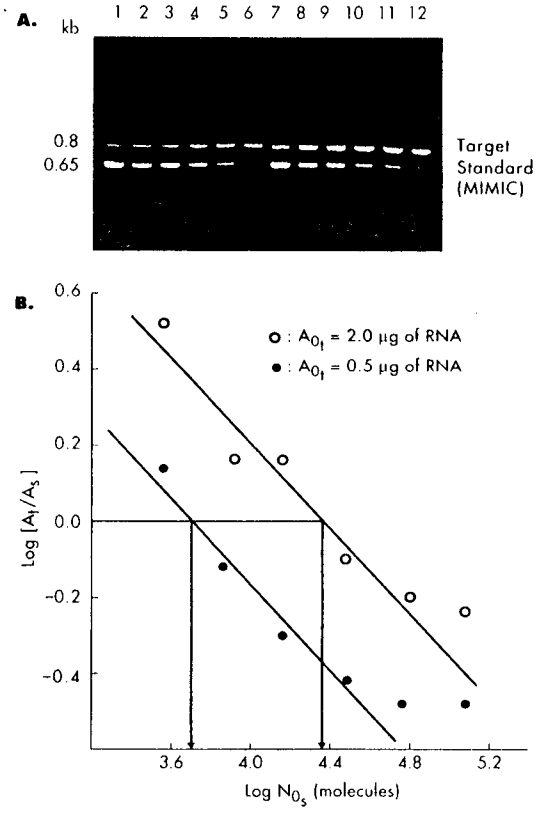

Figure 13. Validation of competitive PCR analysis as a method to determine relative levels of mRNA in two samples. Panel A. Lanes 1–6 and Lanes 7–12: PCR products generated using cDNA templates reverse transcribed from 0.5 µg and 2 µg of human total RNA, respectively. The PCR reactions were carried out using IL-1β-specific primers, in the presence of 2-fold serial dilutions of the IL-1β PCR MIMIC, starting with 20 x 10^{-2} attomoles of MIMIC (in Lanes 1 & 7). α^{32}P-dCTP was included for labeling the products. After 30 amplification cycles, the PCR products were resolved on a 1.6% agarose/EtBr gel. Panel B. The amount of radioactivity present in each of the bands corresponding to the IL-1β target and MIMIC was determined by scintillation counting and the relative amounts of target and MIMIC products were calculated. The Log of the ratios of the two types of products were graphed as a function of the initial amount of MIMIC added to the PCR reactions. The lines were drawn from a linear regression analysis of the data points (excluding the values obtained using the highest amounts of MIMIC).

The initial amount of IL-1β target cDNA present in each reaction was extrapolated from the point on each curve where the amount of amplified target equals the amount of amplified MIMIC (i.e., Log of 1:1=0).The values obtained from the 0.5 µg and 2 µg RNA plots were 4.75 x 10^3 and 22.9 x 10^3 molecules, respectively, indicating a 4.8-fold difference in the initial amount of target. This experiment was repeated three times with similar results. The change in IL-1β mRNA determined from all four experiments ranged from 3.3- to 5.0-fold, with an average of 4.3-fold—very close to the predicted value of 4.0-fold. Thus, competitve PCR using PCR MIMICs can be used to accurately quantitate at least a 4-fold difference in target mRNA levels between different samples.

A Practical Application of PCR MIMICs

As a practical application of this method, competitive RT-PCR was used to study the induction of include macrophage-type nitric oxide synthase (i-NOS) mRNA by lipopolysaccharide (LPS) in a mouse macrophage cell line. An i-NOS primer set and PCR MIMIC were designed to generate PCR products of 550 bp and 300 bp, for target and MIMIC sequences, respectively. Figure 14 illustrates a MIMIC competition experiment with cDNA prepared from macrophage cells before and after a 6-hr LPS treatment. Equal portions of cDNA were amplified in the presence of 4-fold serial dilutions of the PCR MIMIC. The products were then resolved on an agarose/EtBr gel (Figure 14A). In this experiment, the data were quantified by computer imaging of the polaroid film and then plotted (Figure 14B).

The values estimated from the graph for the initial amount of target cDNA in the two samples were 60 and 1,030 cDNA molecules, respectively—a change of 18-fold upon LPS induction. (A correction was made for the difference in size between the MIMIC and target PCR products.) In agreement with these results, it has recently been demonstrated by Northern blot analysis that i-NOS mRNA is induced at least 10-fold following treatment of macrophage cells with LPS (49).

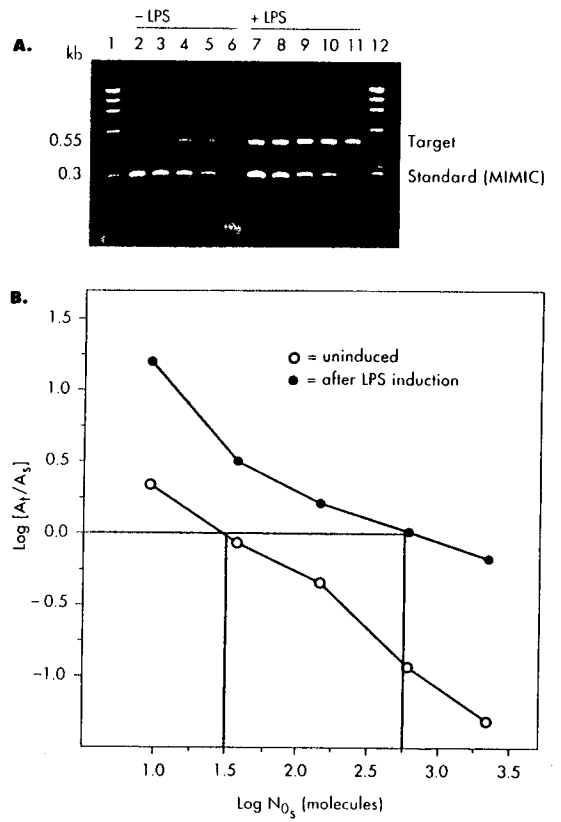

Figure 14. Competitive RT-PCR analysis of inducible macrophage-type (i-NOS) NOS mRNA induction. Total RNA was isolated from mouse RAW 264.7 (i-NOS) macrophage cells before and after a 6-hr stimulation with 10 µg/ml LPS. 2 µg of RNA was used to synthesize cDNA with an oligo(dT) primer. 5% portions of cDNA were amplified with primers specific for mouse i-NOS in the presence of 4-fold serial dilutions of the NOS PCR MIMIC, starting with 10 attomoles (Lanes 2 & 7). Panel A: Following 34 cycles of amplification, 20% portions of the PCR products were resolved on a 1.8% agarose/EtBr gel. Lanes 1 & 12: ϕX174/*Hae* III DNA size markers. Panel B: The relative intensities of the bands corresponding to the target and MIMIC PCR products were quantitated by computer imaging of the polaroid photo of the gel (from Panel A). The relative amounts of target and MIMIC products were calculated after correcting for the difference in size between them. The Log of the ratio of the amount of target to MIMIC PCR products was graphed as a function of the Log of the amount of MIMIC added to the reaction.

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We performed a study of the time course of induction of i-NOS mRNA in LPS-stimulated mouse macrophage cells, using semi-quantitative competitive RT-PCR. In this study, it would have been labor-intensive to perform a MIMIC titration for each of the ten time points, so instead we included equal amounts of the i-NOS MIMIC in each PCR reaction (Figure 15A). In this way it was still possible to correct for amplification efficiency in each reaction and calculate relative changes in mRNA levels. A maximum induction of about 26-fold was observed four hours after LPS treatment (Figure 15B). This was followed by a decrease in i-NOS mRNA levels.

Use of RNA MIMICs

The method for generating competitive PCR MIMICs can be extended to enable generation of heterologous RNA MIMICs to explicitly control for the cDNA synthesis step. To generate an RNA MIMIC, an RNA polymerase promoter and poly A^+ tail can be incorporated into the PCR product using composite primers designed for that purpose. *In vitro* transcription of the PCR product generates synthetic RNAs that contain the target primer sequences and a poly A^+ tail. RNA samples can then be titrated with the RNA MIMIC during reverse transcription. Transcriptional promoters have been successfully incorporated into PCR products via primer sequences (20), and recently, competitive RNA fragments have been generated by this method (50).

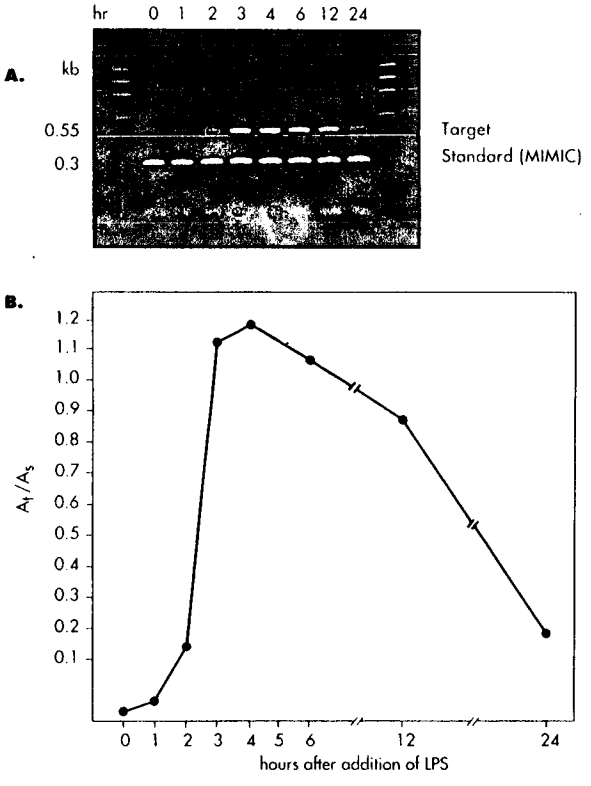

Figure 15. Use of competitive RT-PCR in a semi-quantitative study of the time course of induction of inducible macrophage-type NOS. Mouse RAW 264.7 macrophage cells were treated with LPS (10 µg/ml), and total RNA was isolated from cell samples at various times during the treatment. 2 µg of RNA from each sample was used to synthesize cDNA with an oligo(dT) primer. 5% portions of the cDNA were amplified with primers specific for mouse i-NOS in the presence of a constant amount of NOS MIMIC (10^{-3} attomoles). Panel A: Following 31 cycles of amplification, 20% portions of the PCR products were resolved on a 1.8% agarose/EtBr gel. Panel B: The relative amounts of target and MIMIC PCR products were calculated as described in Figure 14. The ratios of the amount of target to MIMIC PCR products in each sample were graphed as a function of time after addition of LPS.

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Chapter 5

Summary of Quantitative PCR Methods

PCR is an exponential reaction in which small variations in amplification efficiency can yield large changes in the amount of products. In addition, later cycles of PCR exhibit the plateau effect, in which the rate of amplification slows and eventually levels off. These characteristics of PCR can make it difficult to obtain quantitative data. However, if specific conditions and proper controls are used, quantitative information about mRNA levels can be obtained. Of the various quantitative RT-PCR techniques currently in use, competitive PCR is often the method of choice. Competitive PCR is accurate enough to discern differences in mRNA levels as small as 2- to 3-fold or smaller. This is comparable to the accuracy of quantitative methods that use either endogenous or exogenous internal standards in noncompetitive experiments.

Some investigators have observed that careful kinetic analyses can be used to determine initial concentrations of mRNAs by linear regression analysis without internal controls. At least one group, Singer-Sam *et al.* (23), obtained satisfactory results without using either internal controls or kinetic analysis. Nonetheless, many have found it necessary to include internal controls to address the problem of tube-to-tube variation in amplification efficiency. Internal controls can be endogenous mRNA or exogenous mRNA added to the cDNA synthesis reaction. In addition, exogenous standards can be designed with the same or different primer annealing sequences as their target molecules. Each type of internal control has advantages and limitations.

One clear advantage of using endogenous internal mRNA controls is that the yield of RNA and the efficiency of the reverse transcription can vary to some extent without loss of accuracy. However, preliminary studies must be performed to ensure that the endogenous control mRNA does not change during the experiment. This must be tested because many genes, including many housekeeping genes whose expression may seem unrelated to the experimental conditions, are transcriptionally regulated by many different agents. In addition, the data must be collected before the amplification reaction reaches the plateau phase. This can be difficult if the endogenous control gene is expressed at a different level than the target gene or if their relative amplification efficiencies differ greatly.

Exogenous internal standards that share the same primer annealing sequences with the target allow calculation of the absolute amount of target mRNA, as determined by Wang *et al.* (7). A similar method, termed competitive PCR, circumvents many of the disadvantages of the other quantitative methods. Competitive PCR can be used to measure relative changes in mRNA levels as well, for example, in gene regulation studies. However, two conditions must be met to use competitive PCR. One, the molar quantity of the competitor RNA or DNA must be known. (Usually this is not a problem because it can be measured by UV spectrophotometry.) Two, the amplification efficiency of the competitor and target must be identical. This is often true because the standard and target possess the same primer binding sequences. If the standard is a DNA fragment, the efficiency of the reverse transcription also must be considered.

Perhaps the most important advantage of competitive PCR is that useful data can be obtained during the entire course of amplification—even after the reaction has reached the plateau phase. This is not the case for methods using internal standards without competition between the standard and target molecules. Recently, however, Pannetier *et al.* (19) cautioned that competitive PCR may not provide accurate results when the sequences of the target

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and standard molecules are completely different (except for the primer sequences) **and** when the data are collected well after the plateau phase of the reaction. As stated previously, an examination of amplification efficiency is warranted.

We have discussed advantages and limitations of using homologous and heterologous competitor DNA fragments as internal standards for quantitative PCR. In summary, homologous competitor fragments have the same amplification efficiency as their corresponding target but can form heteroduplexes which can complicate the measurement of PCR products. Heterologous competitor fragments, on the other hand, cannot form heteroduplexes, but their amplification efficiencies must be shown to be equal (or very similar to) that of the target. PCR MIMICs, developed at CLONTECH, are nonhomologous competitor fragments that share identical primer binding sequences with their respective targets. We have shown that PCR MIMICs can be amplified with the same efficiency as their corresponding target, thus making competitive PCR using PCR MIMICs a useful and simple method of mRNA quantification.

Conclusions

As we have shown, it is possible to obtain quantitative information about specific mRNA levels using RT-PCR. The ability to obtain accurate measurements of gene expression in small amounts of tissue or in mixed cell populations will considerably expand future applications of PCR, both in research laboratories as well as in clinical settings. For example, we can expect quantitative PCR to be used increasingly in gene expression studies aimed at understanding the basic mechanisms controlling differentiation, development, immunity, and tumorigenesis. In a clinical application, competitive PCR has already been used to quantitate HIV transcripts in patient samples (51). In the future, quantitative RT-PCR can be expected to aid the diagnosis and monitoring of many human diseases.

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Notes

Erratum & Additional References

1. Genomic DNA contamination

a. On page 14 an error was made in describing the RNA template-specific PCR (RS-PCR) method published by Shuldiner *et al.* (20). It does not involve RNase treatment of the RNA, but instead uses a special composite downstream primer which contains two sections: a 3' segment linked to a tagging sequence that becomes incorporated into the cDNA strand during reverse transcription. The tagging sequence is not incorporated into contaminating genomic DNA that may be present. The tagging sequence is subsequently used in the PCR amplification.

A detailed description of RS-PCR is provided by Shuldiner, A. R., Tanner, K., Moore, C. A., & Roth, J. (1991) RNA Template-Specific PCR: An Improved Method that Dramatically Reduces False Positives in RT-PCR. *BioTechniques* **11**:760–763.

b. An additional reference pertaining to DNA contamination involves the use of RNase-free DNA treatment of RNA before reverse transcription: Grillo, M. & Margolis, F. L. (1990) Use of Reverse Transcriptase Polymerase Chain Reaction to Monitor Expression of Intronless Genes. *BioTechniques* **9**:262–268.

2. Incorrect Reference

Reference 7 on page 33 is cited as describing the use of pre-amplification heating to increase yield and specificity. The correct reference number is 15.

TABLE OF CONTENTS

Chapter 1

Introduction and Overview

Control of gene transcription, the process in which a gene's DNA sequence serves as a template for mRNA synthesis, plays a critical role in the multi-step process that regulates gene expression. Gene transcription levels within a cell change in response to a wide variety of signals that occur during cell development, differentiation and normal physiological function. Changes in transcription levels also occur in response to disease and other factors. In turn, these changes in transcription levels cause variations in the steady-state levels of individual mRNAs. Thus, analysis of mRNA levels of a gene is vital in a broad range of research areas.

Traditionally, levels of individual mRNAs have been analyzed by procedures such as Northern blots, RNA dot/slot blots, nuclease protection and *in situ* hybridization. Application of the polymerase chain reaction (PCR) technique provides another method of mRNA analysis. This PCR-based method has been variously termed RNA-PCR (1), RT-PCR (2), RNA phenotyping (3), and Message Amplification Phenotyping (MAPPing) (4). We will use the term RT-PCR throughout this discussion.

The RT-PCR method has become increasingly popular for analysis of gene transcripts, primarily because it is highly sensitive and rapid. A flow chart illustrating the RT-PCR process is shown in Figure 1. RNA is first isolated from tissues or cells

and then used as a template for reverse transcription to complimentary DNA (cDNA). The cDNA in turn is used as the template for PCR, using primers designed to amplify a selected cDNA region. Following PCR, the product is typically analyzed by agarose gel electrophoresis. The amplified cDNA is identified by the size of the PCR product (its "phenotype"), which is predicted from knowledge of the cDNA nucleotide sequence. The PCR product can be further validated by restriction digestion, hybridization or nucleotide sequencing.

The extent of expression of the genes under study can be roughly estimated by knowing the amount of RNA used for the synthesis of cDNA, the amount of cDNA used for PCR, and the number of PCR cycles needed to generate a visible band on an agarose gel. More precise quantitation of individual mRNA levels can be achieved by careful consideration of amplification efficiencies and yields.

We will begin our discussion in Chapter 2 by reviewing the steps involved in RT-PCR, including RNA isolation, cDNA synthesis, and PCR amplification. In Chapter 3, we cover several applications of RT-PCR, including the generation of cDNA probes for Northern gel analysis, detection of alternate splicing of gene transcripts and mRNA quantitation methods. Selected protocols are provided in Chapter 5. This discussion is by no means intended to be complete, and readers are directed to an excellent review on the biochemistry of PCR by W. Bloch (5).

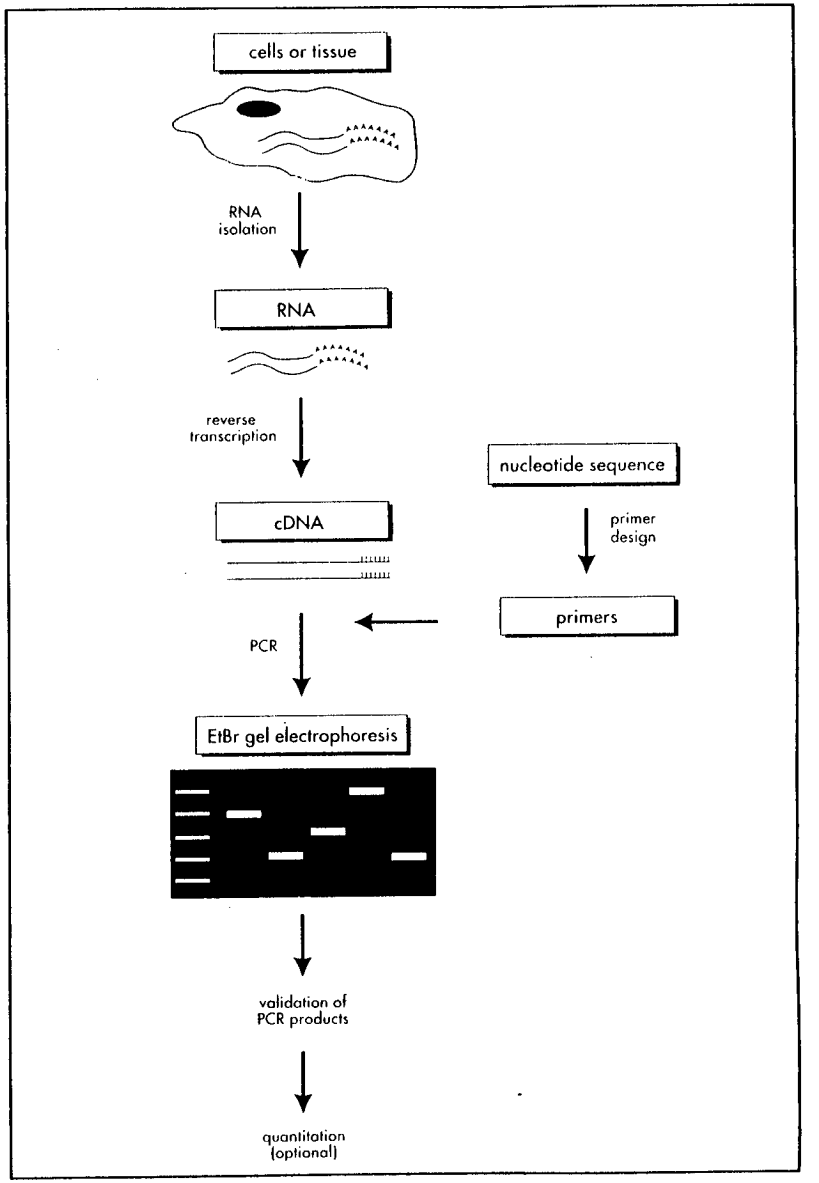


Figure 1. Schematic diagram of the RT-PCR method.

Chapter 2

RT-PCR Method

RNA Isolation

isolation methods

The use of high-quality RNA is critical for the success of RT-PCR analysis. The RNA must not be degraded by ribonucleases, as determined by the intactness of ribosomal (rRNA) bands, and contaminating genomic DNA must be removed. The most common and consistently successful methods for isolating pure, intact total RNA are modifications of the original guanidinium thiocyanate method of Chirgwin, *et al.* (6).

In one such method, tissues or cells are disrupted in high concentrations of guanidinium thiocyanate to rapidly inactivate ribonucleases. The resulting lysate is layered over a CsCl cushion and spun in an ultracentrifuge. The RNA forms a pellet at the bottom of the tube, while protein and DNA remain in or above the CsCl cushion.

Another modified guanidinium thiocyanate method, that does not require an ultracentrifuge, involves coextraction with phenol at reduced pH to remove protein and DNA (7). This is often the method of choice when multiple RNA extractions are performed. Both of these methods are rapid, efficient, and work well for many tissues and cell types. The molecular cloning manual by Sambrook, *et al.* (8) also contains useful information on how to isolate and handle RNA properly. Additionally, several companies offer kits for RNA isolation, including CLONTECH's Magna Poly AAA$^+$ RNA Isolation Kit.

When isolating RNA from small amounts of tissue or cells, a carrier nucleic acid such as tRNA (4) or polyinosinic acid (9) should be added at the beginning of the extraction to facilitate handling of the RNA and to improve yields. To ensure optimal RT-PCR, all RNA preparations should be examined by denaturing agarose gel electrophoresis. If the RNA is intact, it will exhibit clear 28s and 18s rRNA bands, with the 28s band about twice as intense as the 18s band.

RNA handling

Isolated RNA can be stored conveniently as an ethanol precipitate at -20°C, or in aqueous solution at -70°C or below for up to one year without appreciable deterioration. Repeated freeze and thaw cycles should be avoided. Poly A$^+$ RNA isolated from total RNA by oligo(dT) cellulose chromatography (8) can also be used for RT-PCR, although this further purification step is not necessary.

cDNA Synthesis

Reverse transcription

RT enzymes

The cDNA template for RT-PCR is synthesized from RNA by reverse transcription. We have successfully used both avian myoblastosis virus (AMV) and Moloney murine leukemia virus (MMLV) reverse transcriptases with comparable results. It is important to include human placental ribonuclease inhibitor in the cDNA synthesis reaction to obtain maximum yields.

RNase inhibitors

It has been shown that an intact mRNA template remaining after cDNA synthesis can interfere with RT-PCR by competing with the cDNA as the PCR template (10). In most cases, the intrinsic RNase H activity of AMV and MMLV reverse transcriptase is sufficient to degrade most of the remaining mRNA template; however, treatment of the cDNA with RNase H may be needed when using a reverse transcriptase lacking RNase H activity.

cDNA priming

gene-specific priming

There are three ways to prime the mRNA for cDNA synthesis (Figure 2). In the first, a 3' (antisense) gene-specific primer is annealed to the mRNA and extended with reverse transcriptase (Figure 2A). This generates a cDNA template for the 5' (sense) primer, allowing PCR amplification to occur. When priming cDNA with a gene-specific primer, a number of experimental parameters may need to be optimized, including primer concentration and annealing temperature (1).

In the second and third methods, the entire population of mRNA molecules is first converted into cDNA by priming with either oligo(dT) (Figure 2B) or random hexamers (Figure 2C). Two gene-specific PCR primers are then added for amplification. We have had the most success with the latter two methods, which is consistent with their prevalence in the literature.

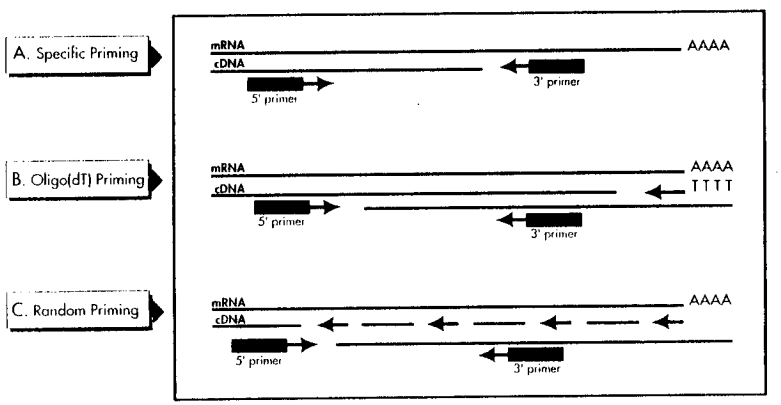


Figure 2. Three methods of priming cDNA. A: Gene-specific method. An antisense gene-specific oligonucleotide is annealed to the mRNA and extended with reverse transcriptase. **B:** Oligo(dT) method. Oligo(dT) oligonucleotides (with lengths between 12-18 nucleotides) are annealed to the Poly A$^+$ tail of mRNA, and the entire population of mRNA molecules used as a template for cDNA synthesis. **C:** Random priming method. Short oligonucleotides (typically hexamers) having all possible nucleotides at each position are annealed randomly to the RNA molecules and used to prime cDNA synthesis.

oligo(dT) and random priming

In our RT-PCR experiments we typically start with oligo(dT) priming, which we have found on average to yield fewer PCR side products than random priming (see Chapter 3). There may be situations, however, when gene-specific or random priming of cDNA may be beneficial. For instance, the reverse transcriptase may fail to fully transcribe an mRNA template if the 5' primer is located greater than about 3Kb from the Poly A$^+$ tail or if secondary structures exist that impede the processivity of the reverse transcriptase.

Several commercial kits are available for synthesizing cDNA, including CLONTECH's 1st-STRAND cDNA Synthesis Kit, specifically designed for RT-PCR.

cDNA synthesis efficiency

In RT-PCR experiments using oligo(dT) or random primers it is advisable to test the reverse transcriptase reaction components and protocol by determining the efficiency of cDNA synthesis. This is easily accomplished by monitoring the incorporation of ^{32}P- labeled dNTPs into TCA-precipitable material (protocol given in Chapter 5). Calculation of cDNA yields is also advisable if several different RNA preparations will be used to compare expression of mRNAs, since the same amount of cDNA template can then be used in each PCR tube. Typical cDNA synthesis yields using MMLV reverse transcriptase and a formula for calculating the efficiency of cDNA synthesis are given in Table 1 on the following page.

single-tube RT-PCR

Several cDNA synthesis reaction mixes have been designed for compatibility with the PCR reaction mix (3). This allows cDNA synthesis and PCR amplification to be carried out in the same tube. We prefer, however, to synthesize cDNA in one 20-30 µl reaction (protocol given in Chapter 5) and then use a 2-3 µl aliquot of the cDNA in each PCR amplification tube. In this way, variability in the cDNA synthesis within each PCR reaction tube is avoided.

Table 1. Typical ss-cDNA synthesis yields using MMLV-reverse transcriptase and various methods of cDNA priming.

METHOD OF PRIMING	INPUT OF RNA	YIELD OF ss-cDNA	% OF TEMPLATE COPIED
oligo (dT)	2 µg Total RNA	30 ng	1.5%
random	0.4 µg Total RNA	186 ng	46.5%
oligo (dT)	1 µg Poly A⁺ RNA	293 ng	29.3%

$$\text{ng cDNA} = \left[\text{fraction of total } {}^{32}\text{P-dNTP incorporated into TCA precipitated cpm determined in a small aliquot.}\right] \left[\text{nmoles each dNTP in the reaction}\right] \left[4\text{dNTPs}\right] \left[\frac{310\text{g}^*}{\text{mole}}\right]$$

*average molecular weight dNTP

NOTE: It is important to assay TCA-precipitable cpm in an aliquot before and after addition of the reverse transcriptase and subtract the values to correct for non-specific TCA precipitation of the labeled dNTP.

Use of Poly A⁺ RNA

We have also examined the use of Poly A⁺ RNA as a template for cDNA. As a result of the enrichment for mRNA, a much smaller volume of the reverse transcription reaction is necessary. In one case, only 1% of the cDNA synthesized from 1 µg of Poly A⁺ RNA was necessary to achieve results comparable to those in Figure 6. The use of total RNA, however, can be advantageous when comparing cDNA derived from several RNA preparations, since one less step (with its potential variability) is necessary to obtain the cDNA template.

PCR Amplification

PCR primer design

We will confine our discussion of primer design to perfect sequence primers, those that exactly match the cDNA template. The location of the primer template within the cDNA sequence is important for several reasons. First, it defines the length of the PCR product. We typically choose primer template locations that yeild products between 400 - 2,000 bp in length. Products smaller than 400 bp require special agarose gel formulations for good resolution and may be obscured by primers and primer artifacts. Products larger than 2,000 bp are less efficiently amplified, due to limitations in enzyme processivity. For example, *Taq* DNA polymerase is not very processive (5) and the enzyme tends to fall off the template during long extensions.

primer location

Second, when the cDNA has been primed with oligo(dT), the primer location defines the distance that the cDNA must be extended from the 3' end of the mRNA to provide the 5' primer template. Because reverse transcriptase has difficulty transcribing long templates, we recommend choosing 5' primer regions not further than 2-3 Kb from the 3' end of the mRNA.

Third, primer locations can be designed to distinguish between PCR products derived from cDNA and products derived from contaminating genomic DNA. We usually try to choose primer sequences that are located on separate exons, so that PCR products derived from genomic DNA will be greater in length.

Finally, it has been shown that secondary structures in mRNA can impede reverse transcription and, therefore, RT-PCR. Thus, avoid designing primers that span a region of the mRNA where stable stem structures (above 14K cal/mol) can form. An RNA folding algorithm has been described for use in RT-PCR by Pallansch (11) to aid in determining such regions.

primer sequence

Typically, PCR primers should be between 22 and 30 nucleotides long and have an A/T content about equal to the G/C content, so that the optimal annealing temperature of both primers is similar. Avoid using primer sequences that can form stable inter- or intra-strand base pairing. It is particularly important that the 3' ends of the primers not be complementary to each other, since much of the

primers would be sequestered as primer dimers (1, 12). In addition, primer sequences must not have sufficient homology with other gene transcripts such that more than one PCR product can be generated.

Several computer software programs have been developed to facilitate primer design (13) and some are available commercially, including CLONTECH's Primer Detective™. Even with these guidelines, the construction of successful PCR primers is empirical, and more than one primer set may need to be tested before a good combination is found. Failure of the primer(s) to work correctly is indicated by no bands on the agarose gel (no product being made) or multiple bands when only one is expected (non-specific amplification).

PCR parameters

reaction components

Basic PCR components include reaction buffer, dNTPs, primers, cDNA template and a thermostable DNA polymerase. The composition of the buffer (e.g., DTT, BSA, $MgCl_2$, gelatin) and the concentration of the dNTPs will vary, depending on the type of enzyme used and, to some extent, the cDNA template and primers. We typically use reaction components recommended for the recombinant AmpliTaq® DNA polymerase (Perkin Elmer-Cetus). A $MgCl_2$ concentration of 1.5 mM is usually satisfactory for most PCR, although some titration is occasionally necessary. Working portions of the reaction components are stored at -20°C and should be discarded after thawing about 10 times.

thermal cycling parameters

Thermal cycling parameters (i.e., times and temperatures for denaturation, annealing and extension) may vary depending on the type of thermocycler used. Typically, denaturation is performed at 94°C for one minute or less, and the polymerase extension step is performed at 72°C for two minutes or less. Perhaps the most critical cycle parameter is the primer annealing temperature. Two excellent discussions of primer length and annealing temperature are provided in references 12 and 14. It has recently been shown that preheating the

PCR reaction tubes before addition of *Taq* polymerase can substantially improve the yield and specificity of the PCR product (15).

In order to examine multiple gene transcripts simultaneously (in the same thermocycler) by RT-PCR, cycle parameters need to be standardized to achieve adequate amplification of all cDNAs. As noted in Chapter 3, comparisons between expression of different transcripts are valid only if the efficiency of the PCR reaction does not plateau. The number of PCR cycles, therefore, should be kept to a minimum.

nucleic acid contamination

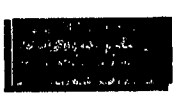
The ability of PCR to amplify DNA sequences by over six orders of magnitude can pose a serious problem when nucleic acid contamination occurs, whether from external sources such as pipette tips, hands or reagents, or from internal sources such as genomic DNA.

In RT-PCR, both nucleic acid and ribonuclease contamination must be controlled. As a general rule, gloves should always be worn and changed frequently, and semi-sterile technique should be adopted. Water used in RNA extraction solutions and in reverse transcriptase reactions should be treated with diethylpyrocarbonate. Water used for PCR amplification should be filter-sterilized, as recirculating water in standard autoclaves can be contaminated with nucleic acids. Many of these precautions are common practice to avoid ribonuclease contamination when handling RNA.

PCR product carry-over

Carry-over of PCR products from previous amplifications must also be minimized. It is a good habit to handle pre- and post-PCR solutions with separate, dedicated pipettors. Special aerosol-free pipet tips are now available from several manufacturers. Whenever possible, perform pre- and post-PCR procedures in separate laboratory areas.

Several chemical means to eliminate the problem of PCR product carry-over have been devised. In one (16), dUTP is substituted for dTTP in the first amplification experiment. In subsequent experiments,



reaction mixtures are treated with uracil N-glycosylase, which cleaves the dUTP-containing nucleotides carried over from the previous experiment and thereby prevents their use as PCR templates. Physical treatment, such as ultraviolet light irradiation, has also been described to decontaminate reagents for PCR (17), although this method is only efficient for large PCR targets.

genomic DNA contamination

Another potential problem during RT-PCR is genomic DNA contamination in the RNA preparation. This is particularly relevant when the target mRNA is expressed at low levels, thus requiring large numbers of amplification cycles. While additional purification steps to completely eliminate genomic DNA may be impractical, there are means to differentiate between amplified cDNA and genomic DNA products. The easiest way is to design the primers such that they span one or more introns within the gene. Thus, PCR products generated from contaminating genomic DNA will be larger than products from cDNA, as shown in Figure 3. This method, termed "intron-differential RT-PCR" (18), can be extended to include digestion at a unique restriction site in the exon that can accentuate differences in gel mobility of PCR products derived from cDNA vs. genomic DNA.

Another method to eliminate genomic DNA contamination is to use a specially designed 3' primer that contains sequences complementary to the last segment of 3' untranslated mRNA sequence including part of the Poly A$^+$ tail (19). In this way, only cDNA derived from Poly A$^+$ RNA can serve as a productive template for PCR. However, this method may prove to be problematic due to the high degree of hetereogeneity found in the 3'-untranslated regions of mRNAs.

In yet another method, referred to as RNA template-specific PCR (RS-PCR) (20), a sample of the RNA to be used for RT-PCR is digested with RNase. PCR products derived from the treated RNA are analyzed by gel electrophoresis; any bands generated will indicate that genomic DNA is present.

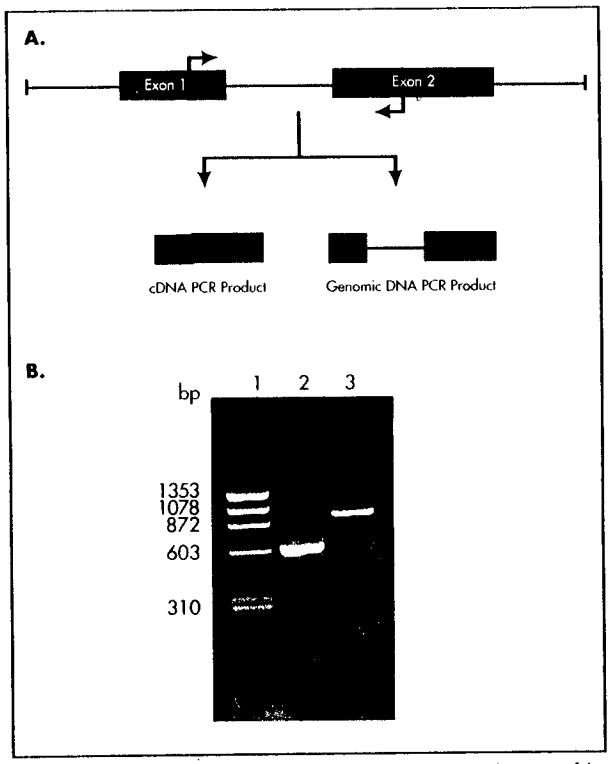


Figure 3. Intron-differential RT-PCR. A: Schematic diagram of the method. If the PCR primers are constructed based on exon sequences separated by one or more introns, RT-PCR products will be obtained with introns spliced out. These PCR products will thus be smaller than PCR products derived from genomic DNA. **B:** Comparison of PCR-amplified human TNF-β from cDNA (Lane 2) and genomic DNA (Lane 3). Lane 1 contains ϕX174/*Hae* III digests as size markers. PCR primers for human TNF-β, which span 2 introns, can distinguish larger genomic contaminants from smaller cDNA products having no intronic sequences.

specificity enhancement

Even under carefully optimized conditions, PCR often results in the generation of non-specific products. This is also true for RT-PCR. Non-specific products may result from amplification of alternatively spliced transcripts or from non-specific primer annealing. Several chemicals, when included in the PCR tube, have been found to increase

specificity by reducing non-specific primer annealing. These include dimethylsulfoxide (DMSO) (21), tetramethylammonium chloride (TMAC) (22), and formamide (23). DMSO has found application in nucleic acid sequencing of double-stranded templates by reducing the rate of re-annealing of the DNA strands. The latter two chemicals have been widely used to increase specificity of nucleic acid hybridization and reduce dependence of hybridization on G/C content.

Verification of mRNA Phenotype

Before any conclusions can be drawn from RT-PCR experiments based solely on the generation of a PCR product of predicted size, the identity of the PCR product must be verified by a second method. This is typically achieved either by partial (or complete) nucleotide sequencing, restriction mapping, or sequence-specific probe hybridization.

Nucleotide sequencing

Obtaining a nucleotide sequence is the most convincing verification method, although it is technically the most demanding and time consuming. The PCR product can be cloned and sequenced by standard methods, or single-stranded products can be obtained by asymmetric PCR (24), strand separation techniques (25), or nuclease digestion (26). There are also methods available to directly sequence double-stranded PCR products (27, 28).

Restriction mapping

Restriction mapping is often the most convenient verification method, accomplished simply by noting the presence of one or more characteristic restriction sites situated between the primer templates. We usually choose an enzyme that cleaves the cDNA fragment only once or twice and yields fragments that can be resolved from each other on an agarose gel. An example of a restriction analysis of an amplified cDNA segment is shown in Figure 4. We have been able to cut unpurified PCR amplified cDNAs with numerous restriction enzymes, listed in Table 2 on the following page. If the reaction buffer must be changed for compatibility with the restriction enzyme, passage of the PCR products through a spin chromatography column provides sufficient purification.

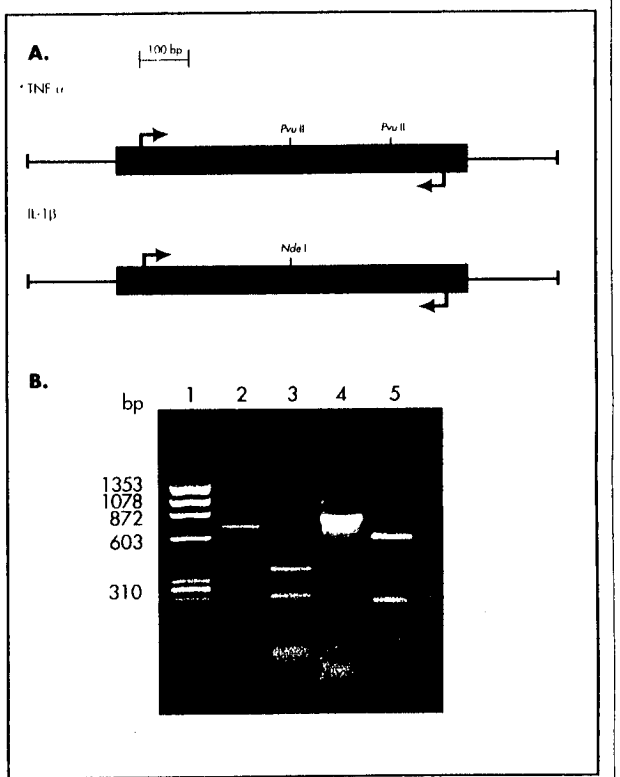


Figure 4. Validation of cytokine MAPPing products by restriction digestion. A: Schematic diagram showing the locations of PCR primers and restriction sites. **B:** Two human cytokine cDNAs, TNF-α (Lane 2) and IL-β (Lane 4), were amplified by RT-PCR using CLONTECH's Cytokine MAPPing™ Amplimers, and digested with *Pvu* II (Lane 3) and *Nde* I (Lane 5), respectively, for 1 hr at 37°C. PCR products and digests were electrophoresed on 2% agarose in TBE. Lane 1 contains ϕX174/*Hae* III digests as size markers. The fragments generated exactly matched those predicted from knowledge of the restriction map of the cDNA and the locations of the primer templates. No purification or exchange of buffers was performed.

Table 2. Examples of restriction enzymes successfully used to digest PCR products without post-PCR purification.

RESTRICTION ENZYME	EXAMPLES OF PCR PRODUCTS CLEAVED
Bam HI	MnSOD
Eco RI	IL-3, IL-4
Hha I	CD4
Nco I	IL-2R
Nde I	IFN-γ
Pvu II	IL-1β, IL-7, TNF-α, TNF-β
Xba I	IL-6
Pst I	tPA, β-Actin
Sac I	β-Actin

Sequence-specific hybridization

Verification of RT-PCR products can also be achieved by successful hybridization of a synthetic oligonucleotide probe that recognizes a unique sequence situated between the PCR primer templates. We typically use antisense, synthetic oligonucleotide probes 30 nucleotides in length to allow for stringent hybridization and washing conditions. Hybridization and analysis can be completed in under 24 hours. Protocols are provided in Chapter 5 and examples of several representative hybridizations are shown in Figure 5.

Although verification by probe hybridization requires synthesis of a third oligonucleotide and a hybridization step, the resulting data can be obtained in the form of an autoradiogram, which can be used to quantitate the amount of PCR product by densitometry and also to differentiate between specific and non-specific PCR products. Further, synthetic oligonucleotide probes with stringent hybridization and washing conditions can be used to differentiate between related gene transcripts, even if their PCR products are similar in size.

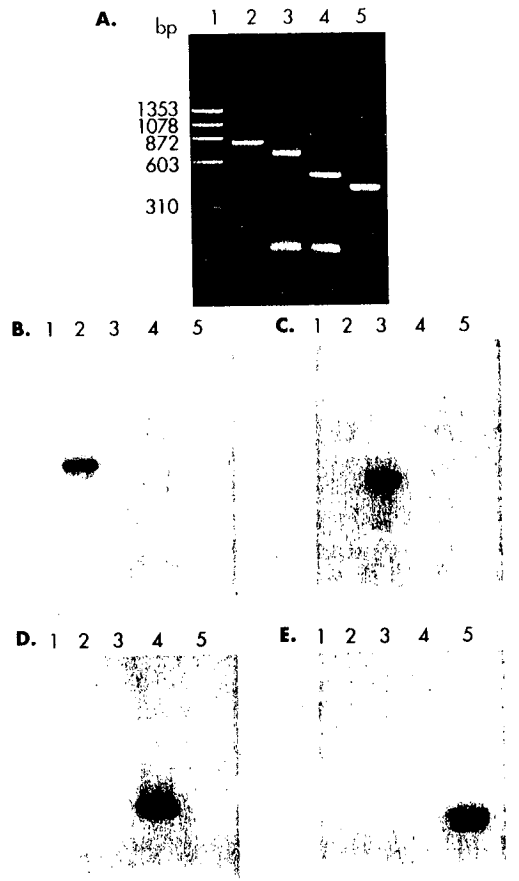


Figure 5. Verification of RT-PCR products by probe hybridization. A: EtBr-agarose gel of cytokine PCR products. Lane 1: ϕX174/*Hae*III markers. Lanes 2-5: PCR products generated using CLONTECH's Cytokine MAPPing™ Amplimers for IL-1β, TNF-α, IFN-γ and IL-2 Receptor, respectively. **B-E:** PCR products from A. were blotted to a nylon membrane and sequentially probed with corresponding Cytokine Probes. In this way, the same blot can be used to verify several PCR products by stripping the blot between each probing. Each probe was end-labeled with γ^{32}P-ATP and T4 Polynucleotide kinase (using CLONTECH's 5'-End DNA Labeling Kit). Each film exposure was less than 15 minutes at room temperature, and the film was hybridized and washed under identical conditions.

Chapter 3

Applications of RT-PCR

Detection of Gene Transcripts from Small Amounts of RNA

Common, traditional methods for detection and analysis of gene transcripts, such as Northern blots and RNA dot/slot blots, require amounts of total RNA in excess of several micrograms, even when examining gene transcripts expressed at high levels. Typically, RNA analyzed by these methods must be further enriched for mRNA by oligo(dT) cellulose chromatography. RT-PCR not only provides a more sensitive method requiring smaller amounts of RNA, but in some cases is the only method that can be used.

For example, the dystrophin gene, defective in patients with muscular dystrophy, is expressed at very low levels (representing only 0.01 - 0.001% of total muscle mRNA), making it difficult to study by conventional methods. RT-PCR was successfully used by Chelly, *et al.* (3) to study levels of this mRNA in clinical samples. The poorly expressed multidrug resistance gene *mdr*-1 has also been studied by RT-PCR, whereas conventional methods were unsuccessful at finding transcripts (29, 30). In some experimental models, genes may be expressed at moderate to high levels, but only in tissues of minute size, such as early mouse embryos. Here again, RT-PCR has proven to be a valuable tool (31).

The exquisite sensitivity of RT-PCR is illustrated in Figure 6A, where the predicted PCR product of 800 bp was clearly visible after 35 cycles of amplification using cDNA synthesized from as little as 8 ng of total RNA using oligo(dT). We also were able to visualize amplified IL-1β cDNA from as little as 60 pg of total RNA using oligo(dT) as a primer when 45 cycles of PCR were performed. In this RNA titration experiment, only 10% of the cDNA synthesized was used in the amplification. This suggests that cDNA derived from as little as 6 picograms of total RNA-the amount of RNA in a single human cell (1)-would be sufficient to detect IL-1β transcripts in human lung.

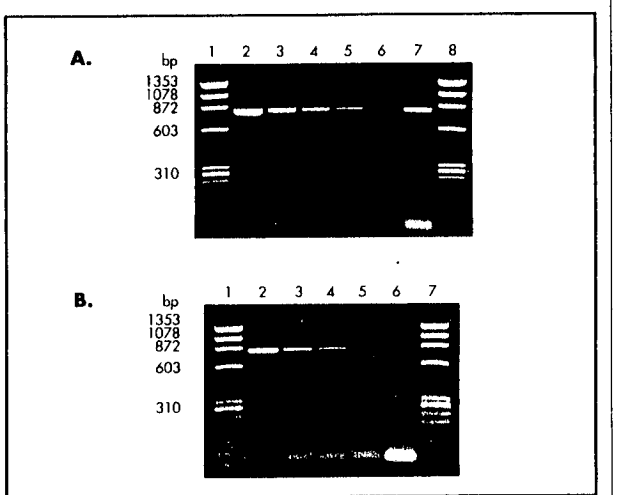


Figure 6. Sensitivity of RT-PCR. Starting with 1 µg of total RNA from human lung, a 5-fold step dilution series was made while maintaining an equal RNA concentration by adding yeast tRNA. cDNA was prepared using either oligo(dT) (A) or random hexamers (B) and a 10% portion of each dilution amplified by PCR. IL-1β, expressed at moderate levels in lung, was used as the target mRNA. 10% of each product was resolved on a 1.6% EtBr agarose gel. **A:** Amplification of cDNA prepared using oligo(dT). Lanes 1 & 8: ϕX174/*Hae* III size markers; Lanes 2-6: IL-1β cDNA products from 1 µg, 0.2 µg, 40 ng, 8 ng and 1.6 ng total RNA, amplified for 35 cycles. Lane 7: IL-1β cDNA products from 60 pg of total RNA, amplified for 45 cycles. **B:** Amplification of cDNA prepared using random hexamers. Lanes 1 & 7: ϕX174/*Hae* III size markers; Lanes 2-6: IL-1β cDNA products from 1 µg, 0.2 µg, 40 ng, 8 ng and 1.6 ng total RNA, amplified for 35 cycles.

oligo(dT) vs. random priming

Amplification of IL-1β from randomly primed cDNA is shown in Figure 6B. Both methods of cDNA priming gave comparable results at 35 cycles, with oligo(dT) priming being slightly more efficient (approximately 5-fold) than random priming. However, when more than 35 cycles were performed, non-specific PCR products were also obtained (data not shown). We have generally found that random-primed cDNA yields more non-specific products than oligo(dT) primed cDNA, perhaps because a high percentage of the cDNA is derived from ribosomal RNA.

Because of the extremely high sensitivity of RT-PCR, one must be cautious when examining the expression of genes in different organs or tissues, since it may not indicate physiological significance. Indeed, Chelly, *et al.* (32) were able to detect by RT-PCR expression of several tissue-specific genes in tissues not expected to express these genes. This phenomenon has been referred to as "illegitimate transcription" or "leaky transcription".

Simultaneous Analysis of Multiple Gene Transcripts

RT-PCR is ideally suited for rapid and simultaneous analysis of several different gene transcripts including cytokines (2, 4), enzymes (33), and oncogenes (34), among others. In addition to needing only a few micrograms of total RNA, one cDNA synthesis can provide enough PCR template to perform a number of amplifications, which usually can be performed in the same thermocycler in less than a few hours.

An example of a multiple transcript analysis by RT-PCR is shown in Figure 7. While such RNA phenotyping by agarose gel electrophoresis is only qualitative at best, several methods exist for semi-quantitation of mRNA expression, as discussed below.

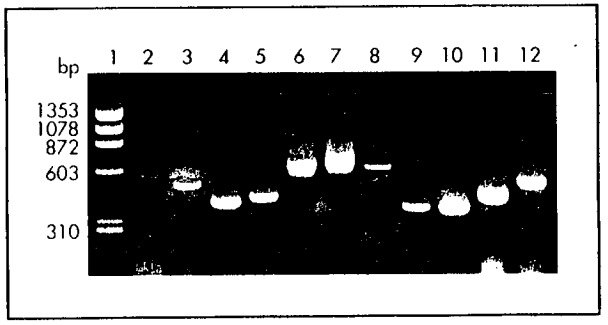


Figure 7. Multiple transcript analysis. Ten different mouse cytokine and cytokine receptor cDNAs, along with cytoplasmic β-actin, were PCR amplified using Cytokine MAPPing™ Amplimers. Total RNA was isolated from activated mouse T-helper cells and cDNA was synthesized using oligo(dT) priming. Following 35 cycles of amplification, 10% of each reaction product was run on a 1.6% agarose gel. The results indicate a wide variation of gel band intensities. Lane 1: φX174/*Hae*III size markers. Lanes 2-12: RT-PCR products of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-2 Receptor, CD4, GM-CSF, IFN-γ, and β-Actin, respectively.

Quantitative Analysis of mRNA Levels

Because RT-PCR requires two enzymatic steps, synthesis of the cDNA template and PCR amplification, both steps must be considered for quantitation of mRNA levels. The cDNA synthesis step will reflect the relative amount of mRNA in the cells or tissue only if two conditions are met: (1) a high proportion of the RNA is intact (see Chapter 2); and (2) cDNA synthesis is efficient for the mRNA under study (see Table 1 for calculating cDNA synthesis efficiency). The PCR amplification step is a complex, exponential process and thus has been the focus of much of the work on quantitative RT-PCR. A discussion of PCR amplification efficiency and quantitation of the resulting PCR products follows. Table 3 on the following page summarizes the steps necessary to quantitate mRNA levels, and methods used for each procedure.

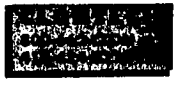


Table 3. Various steps involved in mRNA quantitation by RT-PCR and the methods used for each procedure.

mRNA QUANTITATION STEPS	METHODS USED
Test for intact RNA	Denaturing gel electrophoresis
Test efficiency of cDNA synthesis	^{32}PdNTP incorporation, by TCA precipitation
Test PCR amplification efficiency	Internal standards
Measure PCR products	• ^{32}P-labeled primers or a^{32}PdNTPs (scintillation counter or densitometry of autoradiograms • ^{32}P-labeled probes (blot hybridization) • non-isotopically labeled primers (HPLC, densitometry of fluorescent-stained gels, biotin-streptavidin binding, antibody detection of digoxigenin)

PCR amplification efficiency

Because PCR amplification is an exponential process, small variations in amplification efficiency can drastically affect the yield of products. In addition, the efficiency of PCR decreases at the latter stages of amplification due to depletion of reaction components, diminished enzymatic activity, and accumulation of products. Therefore, any attempt to quantitate mRNA levels by PCR must be limited to the analysis of products generated only during the exponential phase of the amplification. Under these conditions, RT-PCR can yield reasonably precise information about relative changes in mRNA levels.

estimating exponential plateau

The PCR product accumulates exponentially up to a concentration of about 10^{-8} M, then accumulates linearly until about 10^{-7} M. The PCR product concentration is proportional to the starting target DNA, as long as product accumulation remains exponential. The point at which exponential accumulation plateaus can be roughly estimated by noting the point at which continued cycles do not produce significantly increased product yields. As a general rule, a plateau is reached when five additional cycles do not yield at least twice as much product.

A comparison of the yield of PCR-amplified cDNA fragments with the amount of input RNA is shown in Figure 8. A linear relationship was observed, although there was some data scatter. Similar results were obtained in several other experiments. From this data it appears that RT-PCR can detect changes in mRNA levels of two-fold or greater without the use of internal standards. This result is consistent with the more comprehensive study of Singer-Sam, *et al.* (33).

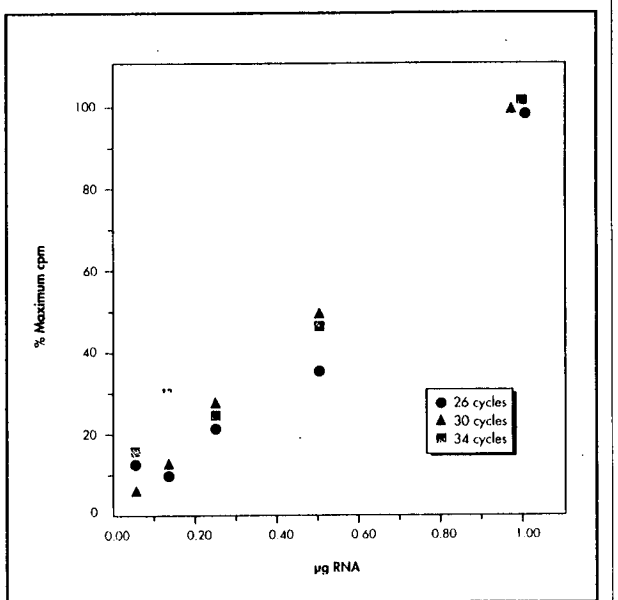


Figure 8. Comparison of PCR product yield with amount of input RNA. Starting with 1 µg total RNA from human lung, two-fold step dilutions of the RNA were reverse transcribed and used as a template for PCR with primers specific to IL-1β. To quantitate the amount of amplified IL-1β cDNA [α^{32}P]-dCTP was included in the PCR reaction. Aliquots of the reaction were taken after 26, 30 and 34 cycles and analyzed on agarose gels. The IL-1β bands were then cut out of the gel and counted in a scintillation counter. The data shown are plotted as a function of the percentage of maximum cpm for each IL-1β sample vs. micrograms of starting RNA. IL-1β products were just visible on the gel at the lowest dilution of 0.03 µg of RNA at 26 cycles (data not shown). In an ideal case, the percentage of maximal IL-1β product vs. input RNA should yield a linear set of data.

internal standards

For more accurate quantitation of mRNA levels, corrections for reaction to reaction variation in amplification efficiency are necessary. This is most commonly performed by inclusion of an internal standard in the cDNA synthesis reaction or PCR amplification. An internal standard is typically a synthetic RNA molecule containing the same primer template sequences as the experimental target. Dilutions of the standard RNA containing known numbers of templates are amplified with the target sample in the same PCR experiment. The internal standard is designed to generate a PCR product of a different size or to delete or add a restriction site to allow differentiation between the amplification products of the target and the internal standard. A standard curve is then constructed. Alternatively, an additional set of primers specific to an invariant control sequence can be added to the same PCR amplification tube. This latter method is sometimes referred to as "multiplex PCR" (31) or "differential PCR" (37). Further information on these methods can be found in references 5, 46 and 47.

Quantitation of PCR-amplified products

PCR products can be quantified by a number of methods, briefly described here. The simplest procedure is to add a radioactively labeled dNTP to the PCR reaction. After gel analysis, the band can be excised and counted in a scintillation counter, or the gel can be dried and an autoradiogram generated and scanned in a densitometer. Another method is to label the 5'-OH group of one or both of the primers with ^{32}P, which is incorporated into the PCR products and then assayed for radioactivity (35).

As discussed in Chapter 2, Southern blot hybridization with synthetic DNA probes can be used both to verify and quantitate PCR generated products, either by densitometry of an autoradiogram or by excising and counting the signal from a hybridization membrane. This method also has the advantage of quantitating only the target product without interference from non-target products or primer-generated artifacts.

Non-radioactive quantitation methods include the use of biotinylated or digoxigenin-labeled primers in conjunction with the appropriate detection methods (36), or HPLC analysis. For an in-depth discussion of the various methods of PCR product quantitation, refer to the review article by W. Bloch (5).

Generation of cDNA Hybridization Probes

In some cases it may be desirable to generate cDNA hybridization probes by RT-PCR. This method circumvents the need to obtain a source of the cloned cDNA, prepare plasmid or lambda DNA, or isolate cDNA inserts.

probe labeling

PCR-generated probes can be prepared by incorporating labeled dNTP either during PCR (38, 39), or after amplification in a separate labeling reaction. After comparing the two methods, we prefer the latter approach for several reasons. For synthesis of probes with high-specific activities it is necessary to keep the concentration of the unlabeled counterpart of the ^{32}P dNTP as low as possible. However, PCR amplification becomes inefficient when any of the dNTPs are in low concentration. Generation of high specific activity probes labeled during PCR amplification has been reported (39), but required the use of a full 250 µCi of labeled dNTP in a single PCR reaction to achieve an adequate concentration of all four dNTPs. This is generally undesirable due to cost. In contrast, one standard PCR amplification can provide nearly 0.5 to 1 µg of cDNA fragment, more than enough material for numerous post-PCR probe preparations using 50-100 ng of cDNA with only 50 µCi of labeled dNTP per probe.

removing unincorporated dNTPs

A critical step in preparing high specific activity probes by PCR amplification is the removal of unincorporated dNTPs from the PCR reaction. For this purpose, we have found spin column chromatography to be very effective and rapid.

Premade columns are available commercially (e.g., CLONTECH's CHROMA SPIN™ Columns) or they can be made in-house following the methods described in Sambrook, *et al.* (8).

Probes with specific activities greater than 0.5 x 10^9 cpm/µg can be routinely prepared from the PCR products by random priming methods. We prefer to use T7 DNA polymerase rather than Klenow fragment, as probes generally have twice the specific activity and the labeling reaction can be completed within 20 minutes. After labeling, spin column chromatography can be used to remove unincorporated dNTPs and small probe fragments.

Several methods are described for non-radioactive labeling of PCR products for use as probes, including the use of biotinylated nucleotides (36) and digoxigenin labeled nucleotides (40). In both cases, the non-radioactively labeled nucleotide can be easily incorporated during PCR amplification.

Figure 9 shows examples of Northern hybridization experiments using cDNA probes generated by PCR. In all cases, less than 10% of the PCR products were used in the labeling reaction. Clearly, PCR generated cDNA fragments can be used as hybridization probes as effectively as purified cDNA inserts obtained from cDNA clones.

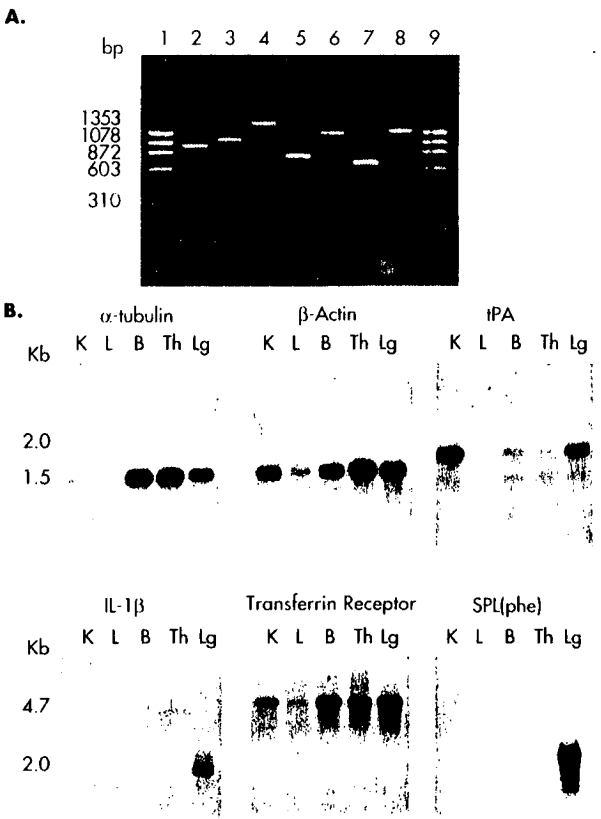


Figure 9. Use of amplified cDNA as hybridization probes. Seven different cDNA fragments ranging in size from 700 - 1,650 bp were amplified by RT-PCR, passed through a CHROMA-SPIN (spin chromatography) column, radioactively labeled, and then used to sequentially probe various Poly A$^+$ RNAs blotted to a nylon membrane. **A:** EtBr-agarose gel of several PCR products. Lanes 1 & 9: ϕX174/*Hae* III size markers. Lanes 2-8: RT-PCR products of α-tubulin, β-actin, tPA, IL-1β, transferrin receptor, TNF-α, and SPL(phe), respectively. **B:** The resulting labeled PCR products were used to sequentially probe one blot containing kidney (K), liver (L), brain (B), thymus (Th), and lung (Lg) Poly A$^+$ RNA (blot was stripped after each probing). Six of the resulting autoradiograms are shown. In all cases, less than 10% of each PCR product was labeled and used as a probe.

Detection of Alternatively Spliced Gene Transcripts

Another application of RT-PCR is the detection of alternatively spliced gene transcripts. If the PCR primers define a segment of mRNA that sometimes contains an exon and sometimes does not, two cDNA fragments of different sizes will be generated. This may show up as a detectable difference in migration of the amplified cDNAs through an agarose gel, as illustrated in Figure 10.

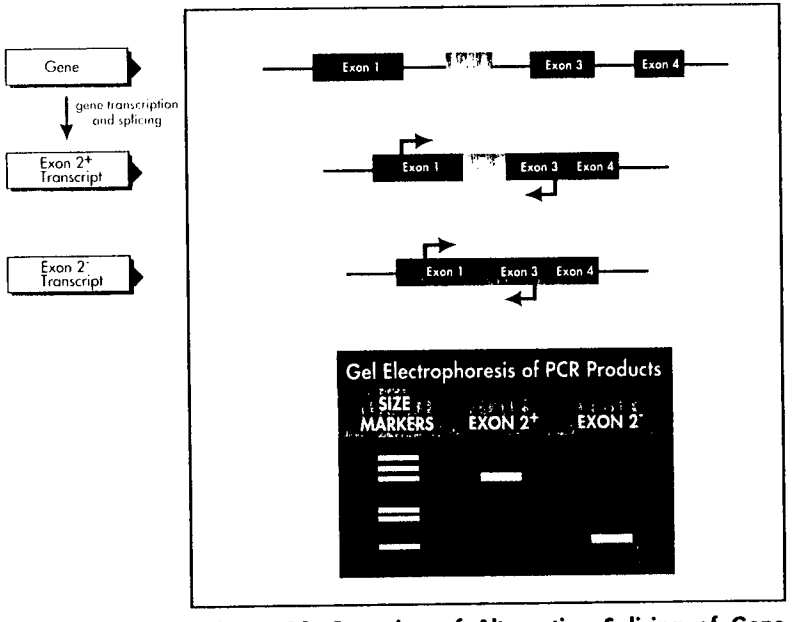


Figure 10. Detection of Alternative Splicing of Gene Transcripts. Schematic of a gene structure containing several introns is shown. If the PCR primers are constructed such that they span an exon that is alternatively spliced (exon 2 in this figure), the RT-PCR products will either contain the exon or will have the exon deleted. Thus, PCR products of two different sizes will be generated, as is graphically represented.

Several examples where altered splicing of gene transcripts has been detected by PCR are described in the literature. Nakabeppu and Nathans (41) observed two PCR products while preparing the coding sequence of the mouse *fos* B cDNA by RT-PCR. One product corresponded in length to the expected *fos* B coding region of 1,250 bp but the other was slightly shorter at 1,100 bp. The authors showed that the smaller *fos* B mRNA (termed Δ *fos* B) encoded a truncated form of *fos* B that inhibited the normal function of the *fos/jun* transcription factors. In another study, Foulkes, *et al.* (42) were able to demonstrate tissue-specific expression of a cAMP-responsive promoter element mRNA with three isoforms, two of which utilized alternative splicing of exons encoding DNA-binding domains. In a third case, Chu, *et al.* (43) found variable deletion of exon 9 in the cystic fibrosis transmembrane conductance regulator gene in bronchial epithelium cells.

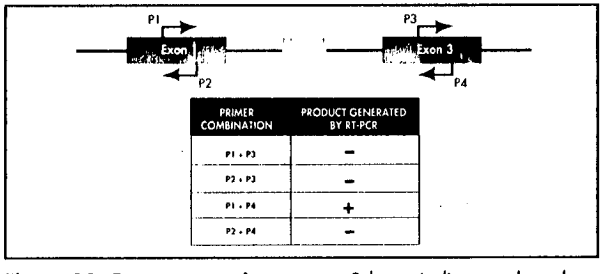
These studies suggest that observation of additional products in RT-PCR experiments should not always be dismissed as non-specific products or PCR artifacts, since they may reveal alternatively spliced gene transcripts.

Exon Connection Assay

RT-PCR can also be used for the assignment of exons in cloned genes. This technique was termed "exon connection" by Vogelstein and co-workers (44) who cloned a 370 Kb DNA fragment from a portion of chromosome 18q suspected to contain a tumor supressor gene. To identify expressed sequences, restriction fragments were subcloned and used in Northern blot experiments with RNA obtained from normal tissues and colon tumor cell lines. No expression was detected using any of the subcloned restriction fragments as probes. For increased sensitivity, RNase protection experiments were performed but this method also failed.

The authors then developed an RT-PCR based exon connection assay (Figure 11). They reasoned that if

the sequences were indeed transcribed into mRNA, a combination of primers constructed from these regions would generate an RT-PCR product. Restriction fragments showing cross-species hybridization (thus, likely to be conserved) were subcloned and partially sequenced. Sequences with open reading frames were used to construct two primers extending in opposite directions. An additional pair of primers was constructed for another region suggesting a transcribed sequence. RNA from tissues expected to express the gene was reverse-transcribed into cDNA with random primers and used as a template for RT-PCR using various primer combinations. This technique succeeded and the authors were able to assign transcribed regions of this tumor suppressor gene. The amplified cDNA segment was cloned and used as a probe to obtain a complete cDNA by conventional methods. The RT-PCR exon connection assay was also used by Vogelstein and co-workers to identify and clone another cDNA suspected to encode a tumor supressor gene located on human chromosome 5q21 (45).



PRIMER COMBINATION	PRODUCT GENERATED BY RT-PCR
P1 + P3	–
P2 + P3	–
P1 + P4	+
P2 + P4	–

Figure 11. Exon connection assay. Schematic diagram shows how the exon connection assay is used to determine whether a novel genomic sequence contains exons (thus encoding a gene transcript) and to establish exon orientation. Sequence information from one or more subclones is used to construct several PCR primers complementary to regions suspected of being exons. For each exon region, two primers are constructed: one in a forward orientation and the other in the opposite orientation. cDNA is then synthesized from RNA obtained from a tissue suspected of expressing the gene, or a tissue such as brain that expresses a large number of different genes. RT-PCR is performed with different combinations of primers, and the PCR products examined on an agarose gel. Successful generation of an RT-PCR product indicates that the particular primer combination is derived from an exon sequence and that the primer templates are on opposite strands of the DNA, thus indicating the relative orientation of the sequence.

Future Perspectives

Improvements in RT-PCR methods and new applications of RT-PCR are appearing with increasing frequency in the literature. We will briefly describe a few of these methods and applications, some of which appeared during the course of preparing this booklet.

In the area of methodological improvements, the use of high performance liquid chromatography (HPLC) appears to offer a useful adjunct to agarose gel electrophoresis for analyzing PCR products, particularly for quantitative PCR (5). Another improvement is the use of pre-amplification heating of the PCR reaction. This has now been shown to increase the yield and specificity of PCR (7). We have found pre-amplification heating to improve the consistency of multiplex PCR (P. Siebert, unpublished observations).

Recent applications of RT-PCR include detection of allele-specific transcription (AST-PCR) (48) and PCR-assisted analysis of cell-free transcription (49). Amplification of DNA from cultured cells and smears directly on microscope glass slides has also been recently discribed (50). It may be possible to extend this method to include reverse transcription with PCR amplification on a glass slide.

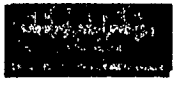
To keep abreast of new information in this rapidly growing field, readers are encouraged to regularly review the literature. We have found several journals particularly informative, including *Nucleic Acids Research* (Oxford University Press), *BioTechniques* (Eaton Publishing Co.), and the new journal *PCR: Methods and Applications* (Cold Spring Harbor Laboratory Press).

Protocols

In this chapter we provide protocols for: (1) cDNA synthesis, (2) quantitating cDNA synthesis by TCA precipitation, and (3) hybridization of probes to PCR products. These protocols are routinely used in our laboratories and are included in several of the products we offer.

Notes and precautions:

- We recommend that you read through each protocol carefully before starting.
- When working with RNA and cDNA, always wear gloves to protect your samples from degradation by nucleases.
- To reduce the risk of DNA contamination of PCR products please read the DNA contamination section in Chapter 3.
- Always wear gloves when handling radioactive materials and dispose of radioactive waste properly.



cDNA Synthesis

We typically synthesize cDNA from a maximum of 2 µg of total RNA in a total volume of 30 µl.

1. Incubate the RNA in 19 µl of DEPC-treated water at 70-80°C for 3 minutes. Spin briefly and place on ice.

2. Add the following components:

Reagent	Amount	Final Conc.
45 µM oligo(dT) 12-18 primer or 100 µM random hexamer primer	1 µl 1 µl	1.5 µM 3.3 µM
5X reaction mix (50 mM Tris-HCl, pH 8.3, 75 mM KCl and 3 mM $MgCl_2$)	6 µl	
dNTP mix, 10 mM each	1.5 µl	0.5 mM
recombinant RNase inhibitor, 50U/µl	0.6 µl	1 U/µl
M-MLV reverse transcriptase, 200 U/µl	2 µl	13.3 U/µl
Total Volume	**30 µl**	

Optional:
^{32}P-dNTP (e.g., dCTP) 5-10 µCi
Adjust water in Step 1. so that total volume is 30 µl.

(M-MLV: recombinant Moloney-Murine Leukemia Virus reverse transcriptase)

3. **Note:** An optional step is to remove a 1 µl aliquot prior to adding enzyme for "minus enzyme" TCA precipitable cpm. Then remove another 1 µl for determination of total ^{32}P-dNTP; dilute into 100 µl of water and count 1 µl of the dilution.

4. Incubate at 42°C for 1 hour. **Optional:** remove 1 µl for "plus enzyme" TCA precipitable cpm.

5. Terminate reaction by incubation at 70-80°C for 10 minutes and spin for 1 minute in a microcentrifuge at 4°C.

6. Remove 2-3 µl for PCR amplification. The remaining cDNA can be stored at -70°C for several months or at -20°C for up to 1 week.

Quantitation of cDNA Synthesis by TCA Precipitation

1. Add 300 µl of a 4 mg/ml solution of fish sperm DNA to two glass test tubes (e.g., 13 x 100 mm) for each cDNA synthesis reaction.

2. Add samples to the tubes and mix. One tube is for the "minus enzyme" sample, the second is for the "plus enzyme" sample.

3. Fill the tubes halfway with cold 5% TCA (trichloroacetic acid, w/v). The tube should become cloudy immediately. There is no need to incubate the tube on ice, and precipitates can be filtered immediately or kept at room temperature until a convenient time for filtration is available.

4. For filtration, use a single 2.4 cm diameter filter holder attached to a side arm Erylenmeyer flask that is connected to a water aspirator. A glass fiber filter, e.g., Whatman 934 AH, is placed in the filter unit. Supply a slight vacuum.

5. Wet the filter with cold 5% TCA and immediately pour the TCA precipitate into the unit. Fill the test tube with 5% TCA and pour into the filter unit to wash the precipitate. Repeat once.

6. Rinse sides of filter unit and filter with cold 95% EtOH. Allow vacuum to continue for a few moments. There is no need to dry the filters under a heat lamp. The few moments of vacuum is sufficient. Place filter in a scintillation vial, add cocktail and count.

7. Calculate the amount of cDNA synthesized by using the following formula (see also Table 1):

$$\text{ng cDNA} = \left[\begin{array}{l}\text{fraction of total } ^{32}\text{P-dNTP} \\ \text{incorporated into TCA pre-} \\ \text{cipitated cpm determined} \\ \text{in a small aliquot.}\end{array}\right] \left[\begin{array}{l}\text{nmoles each} \\ \text{dNTP in the} \\ \text{reaction}\end{array}\right] [4\text{dNTPs}] \left[\begin{array}{l}310\text{g}^{*} \\ \text{mole}\end{array}\right]$$

*average molecular weight dNTP

Hybridization of Oligonucleotide Probes to PCR Products

Southern Blotting Procedure

For multiple probing we recommend a nylon membrane. We typically use 0.2 µm Schleicher & Schuell Nytran (a positively charged nylon). A 0.2 µm pore size is recommended over the standard 0.45 µm size for retention of DNA fragments under 500 bp.

1. Denature DNA in the gel in 0.5 M NaOH, 0.15 M NaCl for 30 min.

2. Neutralize gel in 1.5 M Tris HCl, pH 7.5, 0.15 M NaCl for 30 min.

3. Blot overnight by the capillary method (see Sambrook, *et al.*, reference 8) or by any other method. Note: the denaturation and neutralization buffer may need to be changed for other blotting methods and type of hybridization membrane).

4. Air dry membrane for 30 minutes to 1 hour.

5. Bake at 70-80°C for 1 hour (vacuum is not necessary).

6. Expose blot, DNA side down, on the surface of a UV light box for 1-2 minutes. (Several companies offer special units designed for optimum UV crosslinking.)

7. Heat seal in a plastic bag and store at room temperature until needed. The dry membrane is stable for many months at room temperature when sealed in plastic.

Labeling of Oligonucleotide Probes

1. Combine the following components:

Reagent	Amount
H_2O	16 µl
10X Kinase buffer (0.5M Tris HCl, pH 8.2, 0.1M $MgCl_2$, 50 mM DTT)	2.5 µl
Oligonucleotide probe (10 pmol)	2 µl
γ-^{32}P-ATP (6,000 Ci/mmol)	3.5 µl
T_4 DNA Kinase, 10 units	1 µl
Total Volume	**25 µl**

2. Incubate at 37°C for 30 minutes.

3. Stop the reaction by adding 5 µl 0.5 M EDTA, pH 7.5.

4. Load the entire reaction on a pre-spun CHROMA SPIN-30 column (2 minutes at 4K rpm in a standard variable speed microcentrifuge). Spin again.

5. Remove 1 µl eluent (total approx. 25 µl), dilute 100X and count 1 µl of the dilution. The labeled probe can be stored at -20°C for up to 1 week.

Hybridization

Hybridization conditions are provided for antisense synthetic oligonucleotide probes 30 nucleotides in length with approximately 50% G/C content. Protocols may differ for oligonucleotides either shorter or longer than 30 nucleotides or those with higher or lower G/C content. Refer to the manual by Sambrook, *et al.* (8).

1. Prepare the following Hybridization Mix, then warm mixture to 50°C to dissolve the SDS:

   - 6X SSC (or 5X SSPE)
   - 10 mM EDTA, pH 7.5
   - 2X Denhardts Solution
   - 100 µg/ml sheared and denatured fish sperm DNA
   - 1% SDS

2. Prehybridize the membrane in Hybridization Mix at 55°C for at least 1 hour. Use enough mix to completely cover the membrane.

3. Open plastic bag and add probe to 4 x 10^6 cpm/ml. Seal bag and mix well.

4. Hybridize 16-20 hours at 55°C.

5. Wash #1: Open bag and rinse membrane several times in 2X SSC, 0.05% SDS at room temperature and then continue for 30 minutes with several changes of solution.

6. Wash #2: 2X SSC, 0.05% SDS 20 minutes at 55°C.

7. Remove filter with forceps and shake off excess wash solution (Do not blot dry). Immediately cover with plastic wrap, mount on filter paper with orientation marks and cover with plastic.

8. Expose X-ray film at room temperature without intensifying screen for 5-20 minutes and develop. Store covered membrane at -20°C. The membrane will be stable for many months.

9. To remove the probe from the blot for reprobing, follow the steps below. We have reprobed blots up to 4 times without a noticeable drop in sensitivity.

   a. Place blot in 10 mM EDTA, pH 7.5 warmed to 75-80°C for 20-30 minutes. Monitor probe removal by using a geiger counter.

   b. Blot off moisture and immediately place in a plastic hybridization bag and seal. Store at -20°C until needed.

   c. Prehybridize and hybridize blot as before.

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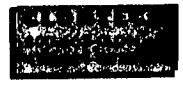

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CLONTECH Related Products

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Total RNA Separator Kit	K1042-1
mRNA Separator Kit	K1040-1
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Poly A$^+$ RNA	several
Total RNA	several
cDNA	
1st-STRAND™ cDNA Synthesis Kit	K1402-1
Recombinant RNase Inhibitor	8405-1
QUICK-Clone™ cDNA	several
cDNA Libraries	several
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CHROMA SPIN™ Columns	several
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PCR-related	
Primer Detective™ software	V1050-1
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Mouse Cytokine MAPPing™ Amplimers	several
Rat Cytokine MAPPing™ Amplimers	several
Detection	
5' End DNA Labeling Kit	K1026-2
Biotin-ON™ Phosphoramidite	5191-1
Cytokine Probes	several
DNA/cDNA Control Probes	several

	Cat. #
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Taq DNA polymerase	8405-1
Heat-TUFF™ DNA polymerase	8400-1
Multi-Pol™ DNA Sequencing System	K1500-1
DNA Parrot data entry system	inquire
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Polymerase Chain Reaction (PCR)

Precautions:

Because the polymerase chain reaction is capable of amplifying as little as a single molecule of DNA, precautions should be taken to guard against sample-to-sample contamination or carry-over of DNA from a previous amplification of the same target.

1. All reagents and solutions used in the PCR must be autoclaved, aliquoted, and stored in an area that is free of PCR-amplification product. Do not use these reagents for other purposes. Discard aliquots of solutions after use.

2. Since the barrels of automatic pipetting devices are common sources of contamination, it is common to prepare and handle reagents using positive-displacement pipettes equipped with autoclaved as well as disposable tips and plungers.

3. The amplification reaction should be set up in a separate room or containment unit. A separate set of supplies and pipetting devices should be dedicated for the specific use of setting up reactions.

4. Quick spin microfuge tubes before opening them,and uncap and close these tubes carefully to prevent aerosol formation. Keep tops tightly closed on all tubes that are not in immediate use.

5. Put on a fresh pair of gloves when beginning work in the area designated for assembly of PCR. Change gloves frequently. Change gloves after handling tubes containing template DNA.

6. Whenever possible, include a positive control (i.e., a PCR reaction tube that contains a small amount of the appropriate target sequence). Always include a negative control that contains all the components of the PCR except the template DNA. Assemble and carry out the negative control after all other reactions have been set up.

LAB 6. In Vitro Amplification of DNA by the Polymerase Chain Reaction Effect of Annealing Temperature on Reaction Efficiency

Reagents and Materials:

Template DNA
10 X amplification buffer
Oligonucleotide primers
Taq DNA polymerase
dNTP mixture
Sterile, nuclease-free H_2O
Mineral oil
Thermal cycler (Perkin-Elmer Cetus or Ericomp)
Micropipettor, autoclaved tubes/tips
Agarose, TAE buffer, ethidium bromide
Disposable gloves

Procedures:

1. In four sterile 0.5-ml microfuge tubes, mix in the template DNA with nuclease-free H_2O as follows:
   a. 5 µl of template DNA (containing from 2.5 pg target DNA and 200 ng irrelevant DNA) and 9.5 µl water. Template is interleukin-1α (3 tubes).
   b. carry-over control (1 tube): no DNA, 14.5 µl pure H_2O

2. Heat to 95°C for 5 min to denature the template DNA, centrifuge briefly (10 sec), then chill on ice. (Alternatively, denaturing step can be set up directly within the thermal cycler.)

3. Add the following reaction reagents to each tube, add reagents in the control tube at last: (We recommend making a master mix for 6 tubes, then adding 9 µl to each tube)

Volume	Reagents	Stock	Final
2.5 µl	10 X amplification buffer	10 X	1 X
4.5 µl	$MgCl_2$, various concentations	25 mM	0.5, 1.5, or 4.5 mM
0.5 µl	dNTP mix solution	2.5 mM	50 µM each
1.0 µl	"upstream" primer	100 ng/µl	4 ng/µl
1.0 µl	"downstream" primer	100 ng/µl	4 ng/µl
1.0 µl	*Taq* DNA polymerase	0.5 U/µl	0.02 U/µl

4. Overlay the reaction mixture with 25 µl of light mineral oil. This prevents evaporation of the sample during repeated cycles of heating and cooling.

5. Place the 0.5-ml tube in the thermal cycler, program the amplification using the following temperature profiles:

Step-cycle,	denaturation	93°C	1.0 min
	primer annealing	50°C	0.5 min
	polymerization	72°C	0.5 min
	perform	20 cycles	
Delay file,		72°C	5.0 min
Soak file,		4°C	

6. Withdraw 10 µl of each amplified sample from the reaction mixture, examine it on a 1.7% agarose gel (electrophoresis at 100V for a mini-gel usually takes 45 min).

Estimated time
PCR set up and cycling: 9:00 - 12:00
Gel electrophoresis: 12:00 - 1:00

LAB 7. In Vitro Amplification of DNA by the Polymerase Chain Reaction Effect of Mg^{2+} and Template Concentrations on Product Yield

Reagents and Materials:

Template DNA
10 X amplification buffer
Oligonucleotide primers
Taq DNA polymerase
dNTP mixture
Sterile, nuclease-free H_2O
Mineral oil
Thermal cycler (Perkin-Elmer Cetus or Ericomp)
Micropipettor, autoclaved tubes/tips
Agarose, TAE buffer, ethidium bromide
Disposable gloves

Procedures:

1. Template is a purified plasmid at 1 fg, 1 pg, or 1 ng per 5 µl in 200 ng/ml sonicated herring sperm DNA in water. Add 5 µl of each concentration to each of three tubes. In a separate tube, add 5 µl water.

2. Prepare three concentrations of $MgCl_2$ from a 25 mM stock so that 4.5 µl added to the reaction tube will give a final concentration of 0.5, 1.5, and 4.5 mM in 25 µl. For each set of DNA sample concentrations, add 4.5 µl $MgCl_2$ at each concentration. Add 4.5 µl 1.5 mM $MgCl_2$ to the tube with water.

3. Add the following reaction reagents to each tube, add reagents in the control tube at last: (We recommend making a master mix for 10 tubes, then adding 6.6 µl to each tube.) Then add 8.9 µl water so each tube contains 25 µl total volume.

Volume	Reagents	Stock	Final
2.5 µl	10 X amplification buffer	10 X	1 X
2 µl	dNTP mix solution	2.5 mM	200 µM each
1.0 µl	"upstream" primer	150 ng/µl	6 ng/µl
1.0 µl	"downstream" primer	150 ng/µl	6 ng/µl
0.125 µl	*Taq* DNA polymerase	5 U/µl	0.025 U/µl

4. Overlay the reaction mixture with 25 µl of light mineral oil. This prevents evaporation of the sample during repeated cycles of heating and cooling.

5. Place the 0.5-ml tube in the thermal cycler, program the amplification using the following temperature profiles:

	denaturation	93°C	4.0 min
Step-cycle,	primer annealing	42°C	30 sec
	polymerization	72°C	45 sec
	denaturation	93°C	45 sec
	perform	25 cycles	
Delay file,		72°C	5.0 min
Soak file,		4°C	

6. Withdraw 10 µl of each amplified sample from the reaction mixture, examine it on a 1% agarose gel (electrophoresis at 75V for 50 min).

Estimated time
PCR set up and cycling: 9:00 -- 12:00
Gel electrophoresis: 12:00 - 1:00

LAB 9. Screening Recombinant Colonies by the Polymerase Chain Reaction

Reagents:

cDNA template
10X Amplification buffer
Oligonucleotide primers
dNTP mixture
Taq DNA polymerase
Sterile H_2O
Mineral oil
DNA thermal cycler
Agarose, TAE buffer, ethidium bromide

Procedures:

1. In a sterile 0.5-ml microfuge tube, add 25 µl of the following cocktail into each tube:

Component	Volume (µl)
10X Amplification buffer*	2.5
dNTP mix solution, 2.5 mM each	0.8
$MgCl_2$, 25 mM	2.0
T7 primer, 150 ng/µl final	1.0
SP6 primer, 150 ng/µl final	1.0
Taq DNA polymerase, 0.025 U final	0.2
Water	17.5

* 100 mM Tris HCl, 500 mM KCl
15 mM $MgCl_2$, 0.1% gelatin, pH 8.3 at room temperature

2. Transfer bacterial colonies to the reaction tube with yellow tips by touching the tip to the colony. Do not attempt to pick a visible portion of the colony. Transfer samples from two independent white colonies and two independent blue colonies. Positive control = IL12p40 plasmid DNA; negative control = nontransformed *E. coli* host cell DH5α.

3. Overlay each reaction sample with 25 µl of light mineral oil.

4. Place the 0.5 ml tube in the thermal cycler.

5. Set the program for amplification using the following temperature profiles:

Step-cycle,	initial denaturation	94°C	4.0 min
	primer annealing	50°C	30 sec
	polymerization	72°C	45 sec
	denaturation	93°C	45 sec
	perform	25 cycles	
Delay file,		72°C	5.0 min
Soak file,		4°C	

6. Analyze 10 µl aliquots on a 1% agarose gel (electrophoresis at 100 V for 30-50 min).

Estimated time

9:00 - 1:00

For research use only. Not for use in *in vitro* diagnostic procedures for clinical diagnosis.

Genius™ System Southern Blotting Protocol (PCR)

This is an abbreviated protocol intended for experienced Genius System users. Please consult the *Genius System User's Guide for Filter Hybridization* for more details if you have not performed this procedure before or if you have any questions.

General Considerations

1. **Hybridization conditions:** DIG-labeled probes can be used under the same hybridization conditions as radiolabeled probes (*e.g.*, in the same hybridization buffer).
2. **Labeling techniques:** While labeling probes with digoxigenin requires slightly more time, it is accomplished with the same methods (*e.g.*, random primed labeling, PCR) as radiolabeling.
3. **Stringency washes:** Blots hybridized with DIG-labeled probes can be subjected to the same stringency washes as radioactive blots.
4. **Probe purity before labeling:** For maximal DIG incorporation and higher yields, probes must be as pure as practically possible (phenol extraction or comparable purification method recommended).
5. **Membrane selection:** The Genius System works best on neutral membranes or membranes with a slightly positive charge. We recommend Boehringer Mannheim Nylon Membranes because they are function tested with the Genius System.
6. **Membrane saturation:** Prior to the addition of chemiluminescent substrates, keep the membrane wet, even to the point of dripping wet. If the membrane dries out or is allowed to become only damp, the chemiluminescent substrate will not spread evenly, and high, blotchy backgrounds will result.
7. **Minimize air exposure:** Minimize exposure of the blot to air during all of the chemiluminescent detection steps.
8. **Hybridization and detection containers:** Use either heat-sealable hybridization bags or freshly washed dishes or trays for both hybridization and detection. Ensure that the membranes remain immersed at all times.

PCR Labeling

1. Assemble a 50 µl PCR labeling reaction with the following components:

Template	Primers	Nucleotides	*Taq* DNA Polymerase	Buffer
1 ng DNA	1.0 µM each upstream and downstream primer	200 µM dATP, dCTP, dGTP 160 µM dTTP, 40 µM DIG-11-dUTP	1.5 Units	1X PCR Buffer (50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM $MgCl_2$)

The optimal ratio of DIG-11-dUTP:TTP must be determined empirically and optimized according to your experimental needs. This protocol uses a 1:5 ratio, which will be useful in multi-copy Southern blot experiments, and possibly, single-copy genomic Southern blot..

2. Mix the reagents and centrifuge briefly.
3. Cycle for 30 to 35 cycles under the following conditions:
   - Denature at 94°C, for 2 minutes
   - Annealing at 52°C, for 2 minutes
   - Extension at 72°C, for 3 minutes
   - (Optional) After final cycle, perform final extension 72°C, for 5 minutes.

Note: As with any amplification, the following may be varied in order to optimize a particular PCR reaction:

- $MgCl_2$ concentration
- concentration of primers
- amount of template DNA
- temperature and time of denature, extension, and annealing steps

Estimating the Yield of DIG-labeled Probe

1. Prepare the following dilution series of DIG-labeled Control DNA in DNA dilution buffer.

Stepwise dilution in RNA dilution buffer	DIG-labeled Control DNA concentration (1 µg/ml)		Final conc. (dilution name)
2 µl/8 µl DNA dilution buffer	5 ng/µl		1 ng/µl (A)
2 µl/18 µl DNA dilution buffer	1 ng/µl	(dilution A)	100 pg/µl (B)
2 µl/18 µl DNA dilution buffer	100 ng/µl	(dilution B)	10 pg/µl (C)
2 µl/18 µl DNA dilution buffer	10 pg/µl	(dilution C)	1 pg/µl (D)
2 µl/18 µl DNA dilution buffer	1 pg/µl	(dilution D)	0.1 pg/µl (E)

2. Spot 1 µl of dilutions B-E onto a positively charged nylon membrane, marking the membrane lightly with a pencil to identify each solution.
3. Make serial ten-fold dilutions of the newly labeled Experimental DNA probe (of unknown starting concentration) in DNA dilution buffer until a 1:10,000 dilution is made (10^{-1} to 10^{-4} dilutions).
4. Spot 1 µl of each of the dilutions made in Step 3 onto the same nylon membrane, marking the membrane lightly with a pencil to identify each dilution.
5. Fix the DNAs to the membrane by cross-linking with UV light or by baking for 30 minutes at +80°C.
6. Wet the membrane with a small amount of Washing buffer.
7. Incubate the membrane in Blocking solution for 1–5 minutes at room temperature.
8. Dilute anti-DIG-alkaline phosphatase antibody 1:5,000 in Blocking solution.
9. Incubate the membrane in the diluted antibody for 5–15 minutes at room temperature.
10. Mix 45 µl NBT solution and 35 µl X-phosphate solution in 10 ml of Detection buffer.
11. Wash the membrane twice, 5 minutes per wash, in Washing buffer at room temperature.
12. Incubate the membrane in Detection buffer for 1 minute. Replace Detection buffer with substrate solution made in Step 10. Expect color development within 20–60 minutes.
13. Match the intensity of color development of the Experimental DNA with the control to approximate the amount of probe.

Probe Hybridization

1. Place blot in a hybridization bag containing 20 ml prehybridization solution per 100 cm^2 of membrane surface area. Seal the bag, after removing air bubbles, and prehybridize at appropriate temperature for 2 hours. The bag should have slightly puffy appearance.
2. When using double-stranded DNA probes, heat in boiling water bath for 10 minutes to denature the DNA before use. Chill on ice.
3. Dilute the probe in prehybridization solution to the correct concentration (5–20 ng/ml).
4. Discard prehybridization solution from bag. Add prehybridization solution containing DIG-labeled probe. Allow probe to hybridize.
5. At the end of hybridization, pour hybridization solution from the bag into a capped tube that can withstand freezing/boiling.
6. Wash the membrane twice, 5 minutes per wash, in 2X wash solution at room temperature.
7. Wash the membrane twice, 15 minutes per wash, in 0.5X wash solution at pre-determined (appropriate) wash temperature.

Chemiluminescent Detection with Lumi-Phos 530

Perform all incubations at room temperature.

1. Equilibrate the membrane in filtered Washing buffer for 1 minute.
2. Allow the Lumi-Phos® 530 or Lumigen® PPD to come to room temperature.
3. Block the membrane in Blocking solution for 60 minutes.
4. Near the end of the blocking period, dilute the anti-DIG-alkaline phosphatase antibody 1:5,000–1:10,000 in Blocking solution. Mix gently by inversion.
5. Pour off the Blocking solution, and incubate the membrane for 30 minutes in the antibody solution.
6. Remove the antibody solution. Gently wash the membrane twice, 15 minutes per wash, in Washing buffer.
7. Pour off Washing buffer, and equilibrate the membrane in Detection buffer for 2 minutes. If using Lumigen PPD, dilute it 1:100 in Detection buffer. Lumi-Phos 530 is pre-diluted; do not dilute.
8. Select application method. Use the single-filter method below for single membrane visualization. See the *User's Guide* for filter-batching method.
   a. Place the membrane between 2 sheets of acetate (plastic page protectors). Ensure that the membrane stays very wet.
   b. Gently lift the top sheet of acetate and, with a sterile pipet, add approximately 0.5 ml (per 100 cm^2) of Lumi-Phos 530 or diluted Lumigen PPD in a line parallel to the side (closest to the fold of the page protector) or the membrane.
   c. Slowly lower the top sheet of acetate allowing the substrate to spread evenly over the entire surface of the membrane. Add additional substrate if the membrane is not completely covered. With a damp lab tissue, gently wipe the top sheet to remove any bubbles present under the membrane and to create a liquid seal around the membrane.
9. In a darkroom, expose the covered membrane to X-ray film at room temperature.
10. When an optimal exposure has been obtained, you may store the membrane in a sealed plastic bag containing TE buffer. If the membrane is to be reprobed, do not allow membrane to dry.

Genius Buffers and Solutions (For the details of making the Hybridization, Maleic acid, or Tris buffers, see the *User's Guide*)

I. For hybridization:

DIG Easy Hyb (Cat. No. 1603 558) A pre-made buffer that offers a convenient ready-to-use, low background, nuclease-free choice for hybridization.

-OR-

Standard hybridization buffer	*Standard hybridization buffer with formamide*
5X SSC	5X SSC
0.1% (w/v) N-lauroylsarcosine	50% Formamide, deionized
0.02% (w/v) SDS	0.1% (w/v) N-lauroylsarcosine
1% Blocking reagent	0.02% (w/v) SDS
	2% Blocking reagent

II. For immunological detection:

DIG Wash and Block Buffer Set (Cat. No. 1585 762), a complete set of ready made washing and blocking solutions. *For use with Northern and Southern blots.*

-OR-

Maleic Acid System
(For use with Northern and Southern blots)

Maleic acid buffer	*Blocking solution*
100 mM maleic acid	Maleic acid
150 mM NaCl:	Dilute the stock solution 1:10 in
adjusted to pH 7.5	Maleic acid Buffer.
(20°C) with solid NaOH.	
Blocking stock solution (10X conc.)	*Washing buffer*
Blocking reagent, 10% (w/v)	Buffer 1 Maleic acid buffer plus
Maleic Acid Buffer	0.3% (v/v) Tween 20

Tris System
(May be used for Southern blots only)

Genius Buffer 1 (Tris)	*Genius Buffer 2* (Blocking solution – Tris)
150 mM NaCl	150 mM NaCl
100 mM Tris-HCl, pH 7.5	100 mM Tris-HCl, pH 7.5
	2% Blocking reagent (vial 11)

III. Additional Solutions (These solutions are for use with all of the above wash and blocking reagent systems.):

Detection buffer	*Color substrate solution*	*TE buffer*
0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5 (20°C)	Always prepare fresh: add 45 µl NBT-solution (vial 9) and 35 µl X-phosphate solution (vial 10) to 10 ml of Detection buffer.	10 mM Tris-HCl 1 mM EDTA: pH 8.0

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Genius™ System User's Guide For Membrane Hybridization

Version 3.0

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Introduction

Welcome to the Genius™ Nonradioactive Nucleic Acid Labeling and Detection System

The Genius System is the most comprehensive, convenient, and effective system for the labeling and detection of DNA, RNA, and oligonucleotides. It also does not require you to learn any new technology. The protocols for labeling with digoxigenin and subsequent detection are based on well-established, widely used methods. DNA, RNA, and oligonucleotide probes are labeled according to the methods (usually enzymatic) used for preparing radioactive probes. Hybridization of digoxigenin-labeled probes (*e.g.*, to target DNA or RNA on a Southern or northern blot) is also carried out according to standard protocols, except that a special blocking reagent is used to eliminate background. The signal on the nucleic acid blot is detected according to the methods developed for western blots. An anti-digoxigenin antibody-alkaline phosphatase conjugate is allowed to bind to the hybridized probe. The signal is then detected with colorimetric or chemiluminescent alkaline phosphatase substrates. If a colorimetric substrate is used, the signal develops directly on the membrane. The signal is detected on an X-ray film (as with ^{32}P or ^{35}S-labeled probes) when a chemiluminescent substrate is used.

DIG Labeling

The use of a nonradioactive probe confers several advantages:

- The technology is safe.
- Probes can be stored for at least a year.
- Hybridization solutions can be reused several times.

The incorporation and spacing of digoxigenin (Figure 1) in DNA, RNA, and oligonucleotides has been optimized for the greatest sensitivity in Boehringer Mannheim's Kits for nucleic acid labeling:

- PCR DIG Probe Synthesis Kit – Digoxigenin-11-dUTP is incorporated by the polymerase chain reaction.
- Genius 1 DNA Labeling and Detection Kit and the Genius 2 DNA Labeling Kit – Digoxigenin-11-dUTP is incorporated by the random-primed labeling method.
- Genius 4 RNA Labeling Kit – The Kit uses T7/SP6-mediated transcription for the synthesis of strand-specific RNA probes.
- Genius 5 Oligonucleotide 3'-End Labeling Kit – Terminal transferase adds a single digoxigenin-11-ddUTP to the 3'-end of the oligonucleotide.
- Genius 6 Oligonucleotide Tailing Kit - Terminal transferase adds a string of digoxigenin-11-dUTP interspersed with unlabeled dATP to the 3'-end of oligonucleotides.
- Genius 8 Oligonucleotide 5'-End Labeling Kit – DIG-NHS ester labels the 5' end.

In addition, protocols have been optimized for nick translation and cDNA synthesis.

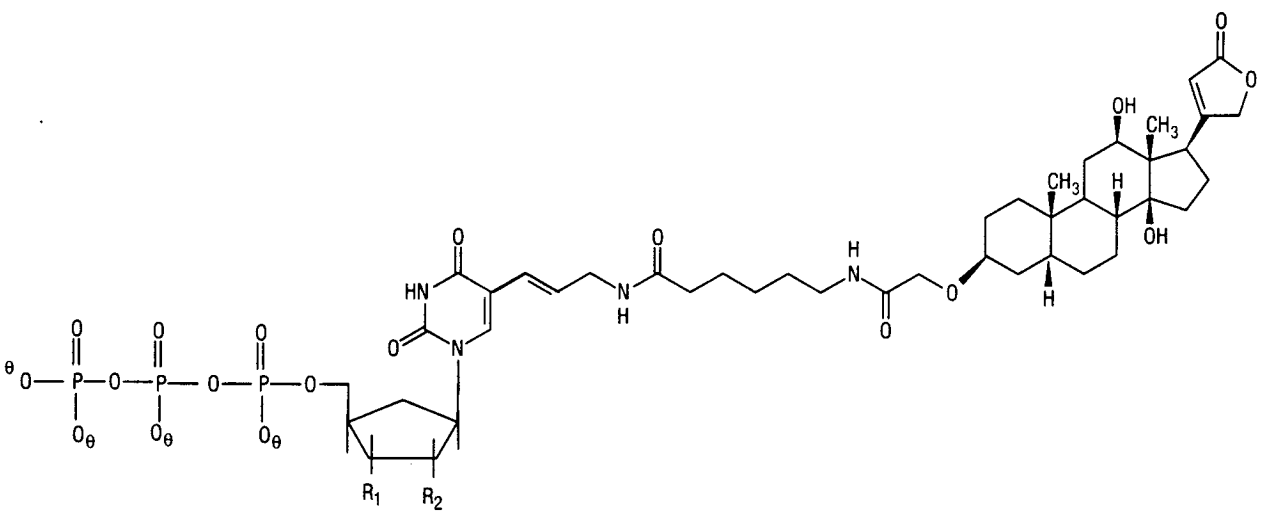

Figure 1. General Structure of DIG-labeled nucleotides. Alkali-stable Digoxigenin-UTP (R_1 = OH, R_2 = OH); Digoxigenin-dUTP (R_1 = OH, R_2 = H); Digoxigenin-ddUTP (R_1 = H, R_2 = H)

As in experiments that use radioactive probes, the yield of the labeling reaction should be estimated to ensure the success of the reaction and to approximate the amount of probe to be used in the hybridization experiment. A simple dot blot method is used to estimate probe yield; the protocol can be found on page 33. Before hybridization, we also recommend that the optimal probe concentration be determined with a "mock hybridization," where various amounts of probe in hybridization solution are hybridized to naked pieces of membrane. This brief procedure ensures high sensitivity and avoids the possibility of high background attributable to a probe concentration that is too high (see page 42).

DIG Detection

Several alternatives are available for the detection of digoxigenin-labeled probes.

- Genius 7 Luminescent Detection Kit – Uses a chemiluminescent alkaline substrate to produce a light signal, which is detected by exposing the membrane to an X-ray film
- Genius 3 Nucleic Acid Detection Kit – Uses the colorimetric substrates NBT and X-phosphate to generate a purple/brown precipitate directly on the membrane
- Multicolor Detection Set – The set comprises three naphthol-AS-phosphate/diazonium salt combinations for the visualization of a green, red, or blue hybridization signal; the dyes are used in combination with the Genius 3 Nucleic Acid Detection Kit or the corresponding single reagents.

There is also a wide range of alternative Anti-digoxigenin conjugates available, such as Anti-digoxigenin-peroxidase, Anti-digoxigenin-gold, Anti-digoxigenin-fluorescein, Anti-digoxigenin-rhodamine, Anti-digoxigenin-AMCA. See Appendix C for a complete listing.

Procedural Similarities and Differences Between the Genius System and Radiolabeling/Autoradiography

There are many procedural similarities between traditional radiolabeling with ^{32}P and labeling nucleic acids with DIG for immunodetection:

- **Hybridization conditions:** DIG-labeled probes can be used under the same hybridization conditions as radiolabeled probes (*e.g.*, in the same hybridization buffer).
  Labeling techniques: While labeling probes with digoxigenin requires slightly more time (see Table 1), it is accomplished with the same methods (*e.g.*, random primed labeling, PCR) as radiolabeling.
  Stringency washes: Blots hybridized with DIG-labeled probes can be subjected to the same stringency washes as radioactive blots.

The quality of your results, however, can be significantly impacted by several differences between nonradioactive nucleic acid labeling with the Genius System and radiolabeling followed by autoradiography (Table 1).

Difference	Genius System	Radioactive Labeling and Detection
Probe Purity Before Labeling	For maximal DIG incorporation and higher yields, probes must be as pure as is practically possible (*e.g.*, DNA that has been washed three times with ethanol has been sufficiently purified).	Probe purification is not required but will increase labeling reaction yield.
Probe Labeling Reaction	Allow the random primed probe labeling reactions to proceed for at least 60 minutes to ensure maximum labeling efficiency and probe yield. Permit scaled-up random primed probe labeling reactions to proceed overnight for best results.	Random primed labeling requires 30 minutes or less to obtain probe incorporation efficiencies of >50% and high-specific activity probes.
Probe Purification After Labeling	After labeling, digoxigenin-labeled probes do not require purification. Unincorporated DIG-labeled nucleotides will not bind to the membranes and are easily removed by wash steps during detection; however, Boehringer Mannheim's Quick Spin™ G-50 Biotinylated DNA Columns may be used if desired (*e.g.*, for *in situ* hybridization applications).	Radiolabeled probes often require purification by mini-columns or spin columns.
Probe Quantification	The amount of DIG-labeled probe must be quantified in a dot blot assay before adding the probe to the hybridization mix. If used at higher-than-recommended concentrations, DIG-labeled probes can cause significant background that is not easily overcome by simply adjusting exposure times.This quantification takes approximately 30 min.	Probe quantification is often performed by TCA precipitation or thin layer chromatography; however, it is not always required because film exposure times can be adjusted to compensate for probe concentrations that are too high or too low.
Membrane Selection	The Genius System works best on neutral membranes or membranes with a slightly positive charge. We recommend Boehringer Mannheim Nylon Membranes because they are function tested with the Genius System.	A variety of charged or neutral membranes can be used. Selection of the best membrane depends on your system and must be determined experimentally.
Membrane Handling	Wearing powderless gloves, handle membranes as little as possible (with forceps) and only at the outer edges of the membrane. This will reduce the incidence and severity of background observed with the chemiluminescent detection.	Handle membranes freely.
Preparation of Membrane for Detection	Requires approximately 12 additional steps. This adds 3 hours, of which 30 minutes is hands-on time for first-time users or 20 minutes for more-experienced users.	No preparation of the membrane is required. Expose directly to X-ray film for 6–72 hr.
Membrane Blocking	The membrane blocking steps prior to immunological detection of the hybridized probe are crucial to optimal results. Insufficient blocking can lead to unacceptable backgrounds and poor sensitivity.	Unnecessary because no antibody is used.
Antibody Addition	The blocking buffer must be completely exchanged with a new, thoroughly mixed buffer containing the appropriate amount of anti-digoxigenin antibody.	Unnecessary because no antibody is used.
Membrane Saturation	Prior to the addition of chemiluminescent substrates, keep the membrane wet, even to the point of dripping wet. If the membrane dries out or is allowed to become only damp, the chemiluminescent substrate will not spread evenly, and high, blotchy backgrounds will result.	Saturation of the membrane is not a concern.
Reuse of probes and hybridization solutions	DIG-labeled probes are stable for at least one year and can be reused repeatedly. In addition, hybridization solutions containing DIG-labeled probes can be stored and reused without a significant loss in sensitivity or increased backgrounds.	^{32}P-labeled probes are unstable and require repeat labeling procedures.

Table 1. Major procedural differences between radiolabeling/autoradiography and the Genius System.

The Power of the Genius System

The Genius Nonradioactive Nucleic Acid Labeling and Detection System can be used for single-copy gene detection on human genomic Southern blots, the detection of unique mRNA species on northern blots, colony and plaque screening, dot/slot blots, and *in situ* hybridization. Examples and protocols for these applications (except for *in situ* hybridizations) can be found throughout the *Genius System User's Guide*. See Figure 2 for an overview of Genius System labeling and detection alternatives. For a comprehensive treatment of nonradioactive *in situ* hybridization, ask for a free copy of Boehringer Mannheim's "Nonradioactive *in situ* Hybridization Manual." The Genius System can also be used for nonradioactive sequencing; see page 67.

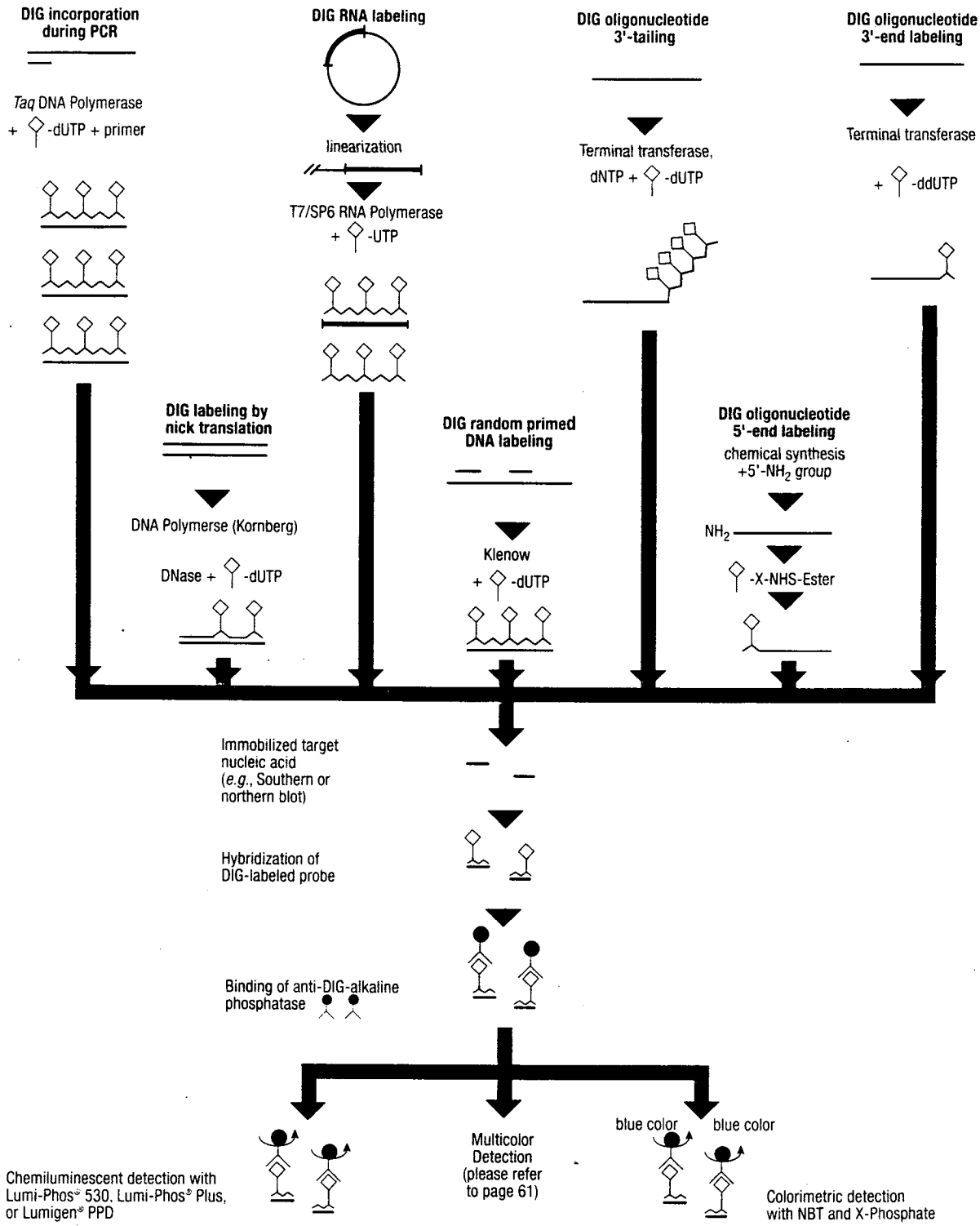

Figure 2. The labeling and detection alternatives offered by the Genius System.

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Lumi-Phos 530 is the subject of patents 4,962,192 and 4,959,182 granted to Lumigen, Inc., Detroit, MI.

An Overview of the Genius™ System User's Guide

Intent of the User's Guide

This manual describes all the digoxigenin labeling methods and all the alkaline phosphatase-based detection assays. All labeling and detection methods are presented in one manual so that users of the Genius System have a convenient reference on which to base their experiments.

What's New About Version 3.0 of the User's Guide

This third version of the *Genius System User's Guide for Membrane Hybridization* includes expanded procedures for labeling DNA probes with DIG by PCR. In addition, it includes many newly released products that increase the convenience of the Genius System, such as the Genius 8 Oligonucleotide 5'-End Labeling Set, DIG Easy Hyb, and the Genius Wash and Block Buffer Set. This version also includes an expanded Troubleshooting/General Laboratory Practices Guide, as well as inclusive procedures for using the Genius Nonradioactive DNA Sequencing Kit.

A significant change to the detection procedures is the replacement of the Tris-based Genius buffers 1 and 2 with maleic acid-based buffers (*e.g.*, Blocking solution, Washing buffer) for detection in all applications. These buffers are easier to prepare than the Tris-based buffers, and they can be DEPC-treated to prevent RNase contamination. Maleic acid is available from Sigma (Cat. No. M0375) or Serva (Cat. No. 28337). For experimental consistency, you may still use the Tris-based buffers (formerly Genius buffer 1 and Genius buffer 2) in any applications except northern blotting if desired. Recipes for the Tris-based buffers are provided in Appendix B.

How to Use the User's Guide

To use the User's Guide, simply select one of the stand-alone sections from each division, choosing one labeling method, one hybridization technique, and one detection method. If you have not already done so, you can then order the required Genius kits or individual Genius System components (listed in the "Required products" table) by consulting Appendix C for complete ordering information. Upon receiving a Genius kit, immediately refer to Appendix A, which contains a complete listing of each kit's components. Use the following flow chart to perform nonradioactive nucleic acid labeling and detection.

Read "General Considerations for Labeling" (page 7)

↓

Perform one labeling procedure (pages 9–32)

DNA Labeling	***Oligonucleotide Labeling***	***RNA Labeling***
• PCR	• Oligonucleotide 3'-end labeling	• *In vitro* transcription
• Random primed	• Oligonucleotide tailing	
• Nick translation	• Oligonucleotide 5'-end labeling	
• Preparing cDNA with DIG-dUTP		

↓

Estimate the yield of DIG-labeled probe (page 33)

↓

Read "General Considerations for Hybridization" (page 41)

↓

Perform one hybridization procedure (page 44–55)

Southern Blot
DNA Dot Blot
Colony/plaque Hybridizations
Northern Blot
RNA Dot Blot

↓

Perform one detection procedure (page 56–66)

Chemiluminescent Detection
Colorimetric Detection
Multicolor Detection

Figure 3. Flow chart for Using the *Genius System User's Guide for Membrane Hybridization.*

Template Purity

In general, the higher the purity of the DNA template, the better the labeling efficiency. We routinely phenol:$CHCl_3$ extract our DNA templates prior to the labeling reaction. In addition, for the random primed DNA labeling method, it is critical that you linearize and heat-denature the template prior to the labeling reaction.

Oligonucleotides should be gel purified or HPLC purified prior to 3'-end labeling or 3' tailing.

Labeling Procedures

When using the Genius System, DNA probes can be labeled by a number of methods. RNA probes are labeled by *in vitro* transcription. Oligonucleotides are labeled by 3'-end labeling, 3' tailing, or 5'-end labeling. Table 2 provides a guide to the different probe-labeling options, the relative sensitivities of the probes produced, and their major applications. Each of the labeling procedures previewed in Table 2 is described in detail in this User's Guide.

Labeling Method	Relative Probe Sensitivity	Application
Incorporation of DIG-dUTP during PCR	+++	• *In situ* hybridization
	+++	• Dot/slot blots
	+++	• Northern blots
	+++	• Southern blots
	+++	• Colony/plaque hybridization
Random primed DNA labeling	+++	• *In situ* hybridization
	++	• Northern blots
	+++	• Southern blots
	+++	• Colony/plaque hybridization
	+++	• Dot/slot blots
Nick translation with DIG-dUTP	+	• *In situ* hybridization
	+	• Northern blots
	++	• Southern blots
	++	• Colony/plaque hybridization
	++	• Dot/slot blots
Preparing cDNA with DIG-dUTP	++	• *In situ* hybridization
	++	• Colony/plaque hybridization
	+++	• Subtraction hybridization
	++	• Northern blots
	+++	• Southern blots
3'-End labeling oligonucleotides	++	• *In situ* hybridization
	+	• Northern blots
	++	• Dot/slot blots
	++	• Southern blots
	++	• Colony/plaque hybridization
3' Tailing oligonucleotides with DIG-dUTP/dATP	++	• Northern blots
	+++	• *In situ* hybridization
	+++	• Dot/slot blots
	++	• Southern blots
	+++	• Colony/plaque hybridization
5'-End labeling oligonucleotides with DIG-NHS ester	N/A	• Primer extension
	N/A	• Sequencing
	++	• Colony/plaque hybridization
	++	• Dot/slot blots
	++	• *In situ* hybridization
Labeling RNA by *in vitro* transcription	+++	• Northern blots
	++	• Southern blots
	+++	• *In situ* hybridization

Table 2. Methods for labeling probes with digoxigenin.

Assay of DIG-labeled Probes

It is important to check the efficiency of each labeling reaction. The purpose of this is to

- **confirm the success of the labeling reaction**
- **estimate the yield of DIG-labeled probe, which must be known for the subsequent hybridization step.**

Probe assay procedures are easy to perform and are described in the section entitled "Estimating the Yield of DIG-labeled Nucleic Acids," which begins on page 33. DIG-labeled controls provided in the Genius kits or sold separately are required for these probe-estimation assays.

Storage of DIG-labeled Probes

One of the major advantages of the Genius System is the long-term stability of DIG-labeled probes. DIG-labeled DNA probe solutions can be stored at –20°C (DIG-labeled RNA probe solutions should be stored at –70°C) for at least 1 year without loss of activity.

Incorporation of Digoxigenin-11-dUTP During PCR

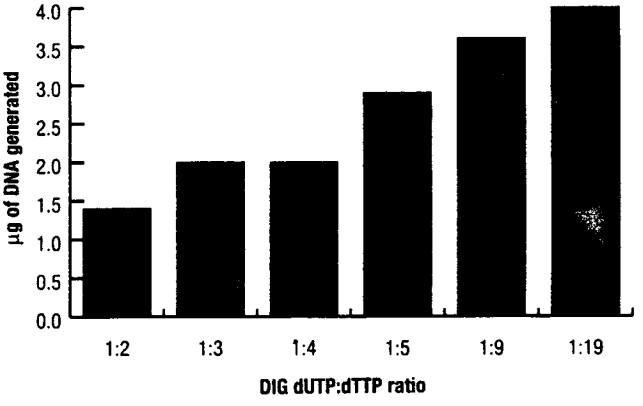
Digoxigenin-11-dUTP (DIG-dUTP) can be incorporated by *Taq* DNA polymerase during polymerase chain reactions. The resulting probes are very sensitive, and the yield from the labeling reaction is quite high. In addition, PCR probe synthesis is a cost-effective use of the DIG-dUTP nucleotide.

Choosing the Right DIG-dUTP:dTTP Ratio for PCR

The amount of DIG-dUTP in the PCR labeling reaction affects both the yield and sensitivity of the probe. When preparing a probe by PCR, it is important to consider the ratio of DIG-dUTP to dTTP in the labeling reaction. Published protocols have described DIG-dUTP:dTTP ratios of 1:2 through 1:20.

We have found that the DIG-dUTP:dTTP ratios of 1:2 through 1:5 are ideal for producing maximally sensitive probes (*e.g.*, for probing genomic Southern blots). Figure 4 demonstrates that decreasing the amount of DIG-dUTP in the amplification reaction increases probe yield.

Figure 4. Effect of DIG-dUTP:dTTP ratios on probe yield. A 1.5 kb *N-ras* fragment was amplified in 100 µl polymerase chain reactions containing 2 U *Taq* DNA polymerase; 25 pmol of each primer; 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3; 200 µM each of dATP, dCTP, dGTP; and various DIG-dUTP:dTTP mixtures at a final combined concentration of 200 µM. A pUC plasmid containing a *N-ras* insert was used as template, and pUC universal primers were used for the amplification. Each PCR also contained 50 µCi of [^{32}P]-dCTP to measure the incorporation of nucleotides. After amplification, samples were spotted onto DE-81 paper, and yields measured as a function of percentage [^{32}P]-dCTP incorporation.



Lesser amounts of DIG-dUTP can be used to make probes for applications that do not demand maximally sensitive probes (*e.g.*, Southern blots containing plasmid DNA, or library screening). DIG-dUTP:dTTP ratios of 1:6 through 1:9 can be used when preparing a probe for these less-demanding applications.

A "trace" amount of DIG-dUTP can be added to a PCR reaction in order to achieve very sensitive detection of the PCR product(s). A 1:19 DIG-dUTP:dTTP ratio, as provided in the PCR DIG Labeling Mix (Cat. No. 1585 550), is useful for direct detection of labeled DNA following PCR amplification.

In summary, the choice of the best DIG-dUTP:dTTP ratio for PCR labeling must be determined empirically and will need to be optimized to obtain the best experimental results. The parameters of probe yield, sensitivity, cost, detection time, and signal-to-noise ratio must be balanced when choosing this ratio. The PCR DIG Probe Synthesis Kit described in this section is provided with pre-mixed nucleotides and features a 1:2 DIG-dUTP:dTTP ratio. This ratio may be used as a starting point for producing probes for applications such as genomic Southern blotting. However, our customers have reported acceptable results with ratios of 1:3, 1:4, or 1:5 in this application. A protocol for probe synthesis with these nucleotide ratios is also provided in this section (page 11).

Generation of DIG-labeled Probes with the PCR DIG Probe Synthesis Kit

The **PCR DIG Probe Synthesis Kit (Cat. No. 1636 090)** provides maximum convenience in generating DIG-labeled probes by PCR because it eliminates the need to prepare a labeling mixture from individual nucleotides. This kit's ready-to-use PCR DIG Probe Synthesis Mix features a 1:2 DIG-11-dUTP:dTTP ratio. This ratio can be used to produce probes for a wide range of filter hybridization applications. Probes generated with this kit can be used to detect single-copy genes in genomic Southern blotting procedures. For applications that do not demand this high level of sensitivity, please refer to page 11 for guidelines on producing alternative nucleotide ratios for probe labeling.

Products required[*†]

	Description	Available From
PCR buffer without $MgCl_2$	100 mM Tris-HCl; 500 mM KCl; pH 8.3 (20°C)	Vial 3, PCR DIG Probe Synthesis Kit
$MgCl_2$ stock solution	25 mM $MgCl_2$	Vial 4, PCR DIG Probe Synthesis Kit
PCR DIG Probe Synthesis Mix	2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.3 mM dTTP, 0.7 mM alkali-labile DIG-11-dUTP, pH 7.0	Vial 2, PCR DIG Probe Synthesis Kit
Taq DNA Polymerase	5 U/µl *Taq* DNA Polymerase	Vial 1, PCR DIG Probe Synthesis Kit
Control template (for control reactions only)	20 pg/µl plasmid DNA in Tris/EDTA buffer, pH 8.0. The 5 kb plasmid contains the cDNA for human tissue type plasminogen activator (tPA).	Vial 5, PCR DIG Probe Synthesis Kit
Control PCR primer mix (for control reactions only)	2 µM of each control PCR primer 1 and 2	Vial 6, PCR DIG Probe Synthesis Kit

Additionally required reagents

In addition to the reagents provided in the PCR DIG Probe Synthesis Kit and your template DNA, you will need the following reagents.

	Description
H_2O	Sterile, distilled water
Upstream Primer	1–10 µM upstream primer solution
Downstream Primer	1–10 µM downstream primer solution
Mineral oil	Mineral oil for overlaying amplification reactions (*e.g.*, Sigma, Cat. No. M5904)

Procedure

❶ Add the following components to a sterile microcentrifuge tube. Place the tube on ice during pipetting.

Reagents	Volume	Volume (Control Reaction)	Final Concentration
H_2O	variable	26.6 µl	——
PCR buffer without $MgCl_2$	5 µl	5 µl	1X (10 mM Tris-HCl, 50 mM KCl)
$MgCl_2$ stock solution	2–10 µl	3 µl	1–5 mM
PCR DIG Probe Synthesis Mix	5 µl	5 µl	200 µM dNTP
Upstream Primer and Downstream Primer **OR**	variable	—	0.1–1 µM of each primer
Control PCR primer mix	—	5 µl	0.2 µM of each primer
Taq DNA Polymerase	0.1–0.5 µl	0.4 µl	0.5–2.5 U/µl
Template DNA **OR**	variable*	—	<1 µg/100 µl
Control template	—	5 µl	2 pg/µl
Total Volume	**50 µl**	**50 µl**	

*Consider the following template DNA amounts as guidelines when generating PCR probes for the detection of single-copy genes: human genomic DNA: 1–100 ng; plasmid DNA: 10–100 pg.

❷ Mix the reagents, and centrifuge briefly to collect the sample at the bottom of the tube.

❸ Overlay with 100 µl mineral oil to reduce evaporation of the mix, and amplify.

Cycling conditions depend on the respective template primers and the thermocycler. For general information about amplification conditions see reference 1. Cycling parameters for the control reaction are as follows:

Denature at 95°C for 7 min before the first cycle.

For 30 cycles:

- Denature at 95°C for 45 sec
- Anneal at 60°C for 1 min
- Extend at 72°C for 2 min.

The control reaction generates an amplification product of 442 bp.

Note: If you are achieving low yields, dilute the PCR DIG Probe Synthesis Mix and add more unlabeled nucleotides to lower the DIG dUTP:dTTP ratio (*e.g.*, to 1:5).

*These products are sold under licensing arrangements with Roche Molecular Systems and The Perkin-Elmer Corporation. Purchase of these products are accompanied by a license to use them in the Polymerase Chain Reaction (PCR) process in conjunction with an Authorized Thermal Cycler.

†The purchase price of these products includes a limited, non-transferable license under U.S. Patents 4,683,202, 4,683,195, and 4,965,188 or their foreign counterparts, owned by Hoffmann-LaRoche Inc. and F. Hoffmann-LaRoche Ltd. ("Roche"), to use only this amount of the product to practice the Polymerase Chain Reaction ("PCR") and related processes described in said patents solely for the research and development activities of the purchaser when this product is used in conjunction with an authorized thermal cycler. No right to perform or offer commercial services of any kind using PCR, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is hereby granted by implication or estoppel. Further information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at The Perkin-Elmer Corporation, 850 Lincoln Center Drive, Foster City, California 94404 or at Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501.

WHAT TO DO NEXT *For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.*

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for in situ hybridization, see page 39.

Reference

1. Erlich, H. A., ed. (1989) *PCR Technology: Principles and Applications for DNA Amplification*, Stockton Press, New York.

Standard Labeling Reaction With Different DIG-dUTP:dTTP Ratios

*Products required**†*

While the PCR DIG Labeling Mix (Cat. No. 1585 550) can be used to label DNA with digoxigenin for direct detection of PCR products, it is not recommended for the generation of DIG-labeled probes because of its 1:19 DIG-dUTP:dTTP ratio. Instead, we recommend the use of the individual reagents *Taq* DNA polymerase, Digoxigenin-11-dUTP, and the Deoxynucleoside triphosphate set (Cat. No. 1277 049). The 100 mM dATP, dCTP, and dGTP preparations must be diluted 1:10 for use in polymerase chain reactions. In addition, the 100 mM dTTP preparation must be diluted 1:100 before adding it to the reaction.

Name in procedure	Description	Available as
100 mM dTTP	100 mM dTTP, lithium salt, solution in Tris-Buffer, pH 7.5 (dilute 1:100 before use)	1. Deoxynucleoside triphosphate set (Cat. No. 1277 049) 2. Cat. No. 1051 482
DIG-dUTP	1 mM digoxigenin-11-dUTP (2'-deoxyuridine-5'-triphosphate, coupled to digoxigenin via an 11-atom spacer arm) in redistilled water	1. alkali-labile, Cat. Nos. 1573 152, 1573 179 2. alkali-stable, Cat. Nos. 1093 088, 1558 706, 1570 013
100 mM dATP	100 mM dATP, lithium salt, solution in Tris-Buffer, pH 7.5 (dilute 1:10 before use)	1. Deoxynucleoside triphosphate set (Cat. No. 1277 049) 2 Cat. No. 1051 440
100 mM dCTP	100 mM dCTP, lithium salt, solution in Tris-Buffer, pH 7.5 (dilute 1:10 before use)	1. Deoxynucleoside triphosphate set (Cat. No. 1277 049) 2. Cat. No. 1051 458
100 mM dGTP	100 mM dGTP, lithium salt, solution in Tris-Buffer, pH 7.5 (dilute 1:10 before use)	1. Deoxynucleoside triphosphate set (Cat. No. 1277 049) 2. Cat. No. 1051 466
10X reaction buffer	100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, (pH 8.3)	1. supplied with *Taq* DNA polymerase 2. Cat. No. 1271 318
Taq DNA polymerase	*Taq* DNA polymerase, in 100 mM potassium phosphate, 10 mM 2-mercaptoethanol, 50% (v/v) glycerol, (pH 8.5)	1. 5 U/µl, Cat. Nos. 1146 165, 1146 173, 1418 432, 1596 594, 1435 094 2. 1 U/µl, Cat. Nos. 1647 679, 1647 687 (both supplied with 10X reaction buffer)

Additionally required reagents

In addition, you will need the following reagents.

H_2O	Sterile, distilled water
Upstream Primer	1–10 µM upstream primer solution
Downstream Primer	1–10 µM downstream primer solution
Mineral oil	Mineral oil for overlaying amplification reactions (*e.g.*, Sigma, Cat. No. M5904)

Procedure for preparing the DIG-dUTP/dTTP mixture

When labeling probes with digoxigenin during PCR, the nucleotides dATP, dCTP, and dGTP should be added at a final concentration of 200 µM, as in standard PCR (*i.e.*, without labeled nucleotides). The combined final concentration of the DIG-dUTP and dTTP must also equal 200 µM.

1. Dilute the 100 mM dTTP preparation 1:100 with H_2O.
   This will eliminate the need to pipet extremely small volumes of dTTP.
2. Use the following chart to prepare the desired nucleotide concentrations for probe amplification.

To prepare 100 µl of stock solution with a DIG-dUTP:dTTP ratio of	Use a 1 mM DIG-dUTP volume of	Use a 1 mM dTTP volume of
1:2 (66 mM:133 mM)	33 µl	67 µl
1:3 (50 mM:150 mM)	25 µl	75 µl
1:4 (40 mM:160 mM)	20 µl	80 µl
1:5 (33 mM:166 mM)	17 µl	83 µl
1:9 (20 mM:180 mM)	10 µl	90 µl
1:19 (10 mM:190 mM)*	5 µl	95 µl

*Probes generated with this ratio are only recommended for applications in which the target nucleic acid is extremely abundant, such as certain colony screenings or PCR dot blots.

An aliquot of the resulting dTTP/DIG-dUTP mixture can be used immediately, and the rest stored at –20°C for subsequent experiments.

Procedure for amplification reaction

1. Dilute the 100 mM dATP, dCTP, and dGTP preparations 1:10 with H_2O.

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†The purchase price of these products includes a limited, non-transferable license under U.S. Patents 4,683,202, 4,683,195, and 4,965,188 or their foreign counterparts, owned by Hoffmann-LaRoche Inc. and F. Hoffmann-LaRoche Ltd. ("Roche"), to use only this amount of the product to practice the Polymerase Chain Reaction ("PCR") and related processes described in said patents solely for the research and development activities of the purchaser when this product is used in conjunction with an authorized thermal cycler. No right to perform or offer commercial services of any kind using PCR, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is hereby granted by implication or estoppel. Further information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at The Perkin-Elmer Corporation, 850 Lincoln Center Drive, Foster City, California 94404 or at Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501.

❷ Prepare an amplification mixture by adding reagents to a sterile microfuge tube in the following order:

Reagent	Volume/50 µl reaction	Volume/100 µl reaction	Final Concentration
10X reaction buffer	5 µl	10 µl	1X (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$)
10 mM dATP	1 µl	2 µl	200 µM
10 mM dGTP	1 µl	2 µl	200 µM
10 mM dCTP	1 µl	2 µl	200 µM
DIG-dUTP:dTTP mixture (prepared above)	10 µl	20 µl	200 µM total (DIG-dUTP+dTTP)
Upstream Primer	0.3–2.5 µl	0.5–5.0 µl	0.1–1.0 µM
Downstream Primer	0.3–2.5 µl	0.5–5.0 µl	0.1–1.0 µM
5 U/µl *Taq* DNA Polymerase		0.2–0.5 µl	0.5 µl 2.5 U/100 µl
OR			
1 U/µl *Taq* DNA Polymerase		1.5 µl	2.5 µl 2.5 µl/100 µl
H_2O	variable	variable	———
Template DNA	variable*	variable*	<1 µg/100 µl
Total Volume	**50 µl**	**100 µl**	

*Consider the following template DNA amounts as guidelines when generating PCR probes for the detection of single-copy genes: human genomic DNA: 1–100 ng; plasmid DNA: 10–100 pg.

To ensure that your polymerase chain reaction conditions are correct, we encourage you to always include a control PCR reaction tube in which DIG-dUTP is omitted (*i.e.*, use dTTP at 200 µM). If the desired fragment is not amplified, this reaction will help determine if lower dilutions of DIG are necessary, or if other conditions should be altered.

❸ Mix the reagents, and centrifuge the tube briefly to collect the sample at the bottom of the tube.

❹ Add 100 µl Mineral oil to the top of the mixture to reduce evaporation, and amplify.

Amplification parameters depend greatly on the template, primers, and amplification apparatus used. For general information on amplification conditions, see reference 1. Typically, the DNA is amplified through 25–35 cycles of denaturation, annealing, and polymerization under the following conditions:

- Denature at 94°C for 2 minutes
- Anneal at 52°C for 2 minutes
- Extend at 72°C for 3 minutes.
- (Optional) After the final cycle, perform final extension at 72°C for 5 minutes.

WHAT TO DO NEXT *For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.*

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for in situ hybridization, see page 38.

Reference

1. Erlich, H. A., ed. (1989) *PCR Technology: Principles and Applications for DNA Amplification*, Stockton Press, New York.

Random Primed DNA Labeling

DNA can be labeled with digoxigenin-11-dUTP using the random primed method. For optimal results, the template DNA should be linearized and purified by at least one phenol/chloroform extraction and ethanol precipitation prior to labeling. Templates of 100–10,000 bp label efficiently and produce probes with maximal sensitivity; therefore, templates >10 kb should be restriction-digested prior to labeling. For genomic Southern hybridizations, we recommend that you separate the insert from vector sequences before labeling.

Standard Random Primed DNA Labeling Reaction

The aim of the standard Random Primed DNA labeling reaction is to produce a sufficient amount of a digoxigenin-labeled probe in the shortest amount of time (1 hour); this 20 µl reaction will yield a minimum of 260 ng of digoxigenin-labeled probe from 1 µg of DNA template (see Table 3). In this standard reaction, one digoxigenin molecule is incorporated in every 20–25 nucleotides.

Products required

Most of the reagents required for random primed labeling are available separately, in the **Genius 1 DNA Labeling and Detection Kit (Cat. No. 1093 657)**, or in the **Genius 2 DNA Labeling Kit (Cat. No. 1175 033)**.

	Description	Available in
Hexanucleotide mixture (10X)	62.5 A_{260} units/ml (1.56 mg/ml) random hexanucleotides, 500 mM Tris-HCl, 100 mM $MgCl_2$, 1 mM Dithioerythritol (DTE), 2 mg/ml BSA; pH 7.2	1. Vial 5, Genius 1 Kit 2. Vial 5, Genius 2 Kit 3. Hexanucleotide Mixture (Cat. No. 1277 081)
dNTP labeling mixture (10X)	1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM alkali-labile DIG-dUTP; pH 6.5	1. Vial 6, Genius 1 Kit 2. Vial 6, Genius 2 Kit 3. DIG DNA Labeling Mixture (Cat. No. 1277 065)
Klenow enzyme, labeling grade	2 units/µl DNA Polymerase I (Klenow enzyme, large fragment), labeling grade, from *E. coli*	1. Vial 7, Genius 1 Kit 2. Vial 7, Genius 2 Kit 3. Cat. Nos. 1008 404, 1008 412
Unlabeled Control DNA 2 (for control reaction only)	200 mg/ml pBR328 that has been linearized by *Eco* RI	1. Vial 2, Genius 1 Kit 2. Vial 2, Genius 2 Kit

Additionally required solutions

In addition to the products above and your DNA template, you will need the following solutions.

	Description
H_2O	Sterile, distilled water
EDTA	200 mM EDTA, pH 8.0

Procedure

1. Dilute 500 ng–3 µg DNA template in H_2O to a total volume of 15 µl (for a final concentration of 0.5–150 µg/ml). For control reactions, mix 5 µl Unlabeled Control DNA 2 and 10 µl H_2O
2. Heat-denature the DNA template in a boiling water bath for 10 minutes, and quickly chill it on dry ice/Ethanol for 30 seconds before use.
   We have found that denaturation using a heating block is less effective and may result in lowered labeling efficiency.
3. Add 2 µl Hexanucleotide mixture (10X) and 2 µl dNTP labeling mixture (10X) to the tube (on ice).
4. Thaw the tube's DNA template, Hexanucleotide mixture (10X), dNTP labeling mixture. Then add 1 µl Klenow enzyme, labeling grade, for a final concentration of 100 U/ml, and mix.
5. Incubate the reaction tube at +37°C for at least 60 minutes.
   Longer incubations (up to 20 hours) will increase the yield of DIG-labeled DNA (Table 3).
6. Add 2 µl EDTA to the reaction tube.
   This terminates the labeling reaction.

Incubation Period	Amount of template DNA per labeling reaction					
	10 ng	30 ng	100 ng	300 ng	1,000 ng	3,000 ng
1 Hour	15 ng	30 ng	60 ng	120 ng	260 ng	530 ng
20 Hours	50 ng	120 ng	260 ng	500 ng	780 ng	890 ng

Table 3. Effect of template amount and labeling time on probe yield. The amount of synthesized DIG-labeled DNA increases with the amount of DNA template in the labeling reaction and the length of the incubation time at +37°C. Yields may vary from this example because of template purity, sequence, etc.

WHAT TO DO NEXT *For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.*

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for **in situ** *hybridization, see page 38.*

Random Primed Labeling with High Prime Digoxigenin DNA Labeling Mix

The **High Prime Digoxigenin DNA Labeling Mix (Cat. No. 1585 606)** offers a convenient alternative to random primed labeling with the Genius 1 Kit, Genius 2 Kit, or individual labeling reagents. Sensitive DIG-labeled probes can be generated easily with this 5X concentrated labeling mixture of random hexamers, dNTP mix containing alkali-labile digoxigenin-11-dUTP, labeling-grade Klenow enzyme, and an optimized reaction buffer. High Prime Digoxigenin DNA Labeling Mix minimizes the hands-on time required to label DNA probes and eliminates most of the pipetting and mixing of reagents and buffers.

Products required

Name in procedure	Description	Available as
DIG-High Prime	High Prime Digoxigenin DNA Labeling Mix (random hexamers, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM alkali-labile digoxigenin-11-dUTP, 1 U/µl labeling-grade Klenow enzyme,5X reaction buffer) in 50% (v/v) glycerol	Cat. No. 1585 606

Additionally required solutions

In addition to High Prime Digoxigenin DNA Labeling Mix and your DNA template, you will need the following solutions.

	Description
H_2O	Sterile, distilled water
EDTA	200 mM EDTA, pH 8.0

Procedure

1. Dilute 500 ng–3 µg DNA template (linear or supercoiled) in H_2O for a total volume of 16 µl.
2. Heat-denature the DNA template in a boiling water bath for 10 minutes, and quickly chill it on dry ice/Ethanol for 30 seconds before use.

   We have found that denaturation using a heating block is less effective and may result in lowered labeling efficiency.
3. Add 4 µl DIG-High Prime, thaw on ice, mix, and centrifuge briefly.

4. Incubate the reaction tube at +37°C for at least 60 minutes.
   Longer incubations (up to 20 hours) will increase the yield of DIG-labeled DNA.
5. Add 2 µl EDTA to the reaction tube.
   This terminates the labeling reaction.

WHAT TO DO NEXT *For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.*

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for in situ hybridization, see page 38.

Nick Translation with Digoxigenin-11-dUTP

Nick translation is a well-established technique for labeling DNA probes. The reaction uses DNase I to create single-stranded nicks in double-stranded DNA. The 5'-3' exonuclease activity of *E. coli* DNA polymerase I enters the nicks and removes stretches of single-stranded DNA; the degraded DNA is then replaced with labeled deoxyribonucleotides by the 5'-3' polymerase activity of the polymerase (1).

For routine blotting experiments, random-primed DNA labeling has perhaps overshadowed nick translation because of the higher specific activities obtained. However, nick translation is an especially useful labeling method for *in situ* hybridization experiments because it allows the lengths of the labeled DNA fragments to be controlled. Probe size is a critical parameter in *in situ* hybridization experiments because the probe has to be small enough to penetrate the tissue or cells.

Probe lengths of 200–500 bp are well suited for *in situ* hybridization experiments, and such probe lengths are readily obtained with the nick translation protocol below. The procedure was originally described by Rigby *et al.* (1) and tested for nucleotide analogues by Langer *et al.* (2).

Standard Labeling Reaction

Products required

The reagents for DIG labeling by nick translation must be purchased separately.

Reagents	Description	Available as
0.5 mM dATP	sold as 100 mM dATP, lithium salt, solution, (dilute 1:200 before use)	Cat. No. 1051 440
0.5 mM dCTP	sold as 100 mM dCTP, lithium salt, solution, (dilute 1:200 before use)	Cat. No. 1051 458
0.5 mM dGTP	sold as 100 mM dGTP, lithium salt, solution, (dilute 1:200 before use)	Cat. No. 1051 466
0.5 mM dTTP	sold as 100 mM dTTP, lithium salt, solution, (dilute 1:200 before use)	Cat. No. 1051 482
DIG-dUTP	1 mM digoxigenin-11-dUTP (2'-deoxyuridine-5'-triphosphate, coupled to digoxigenin via an 11-atom spacer arm) in redistilled water	1. alkali-labile, Cat. Nos. 1573 152, 1573 179 2. alkali-stable, Cat. Nos. 1093 088, 1558 706, 1570 013
DNase I	2000 U/mg DNase I (Grade II), lyophilized	Cat. No. 104 159
DNA Polymerase I	1 mg/ml DNA Polymerase I in 50 mM potassium phosphate; 0.25 mM dithiothreitol; 50% (v/w) glycerol; pH approx. 7.0	1. Cat. No. 104 485 2. Cat. No. 642 711 (endonuclease-free)

Additionally required solutions

In addition, you will need the following solutions.

Description
10X reaction buffer	500 mM Tris-HCl, pH 7.8; 50 mM $MgCl_2$; 0.5 mg/ml BSA, nuclease-free
DTT	100 mM Dithiothreitol (DTT)
H_2O	Sterile, distilled H_2O
EDTA	500 mM EDTA, pH 7.4

Procedure

❶ Add reagents to a sterile microfuge tube (on ice) in the following order:

Reagent	Volume	Final Concentration
10X reaction buffer	5 µl	1X
100 mM DTT	5 µl	10 mM
Deoxynucleotide triphosphates (0.5 mM dATP, dGTP, dCTP each)	4 µl	40 µM
0.5 mM dTTP	1 µl	10 µM
1 mM DIG-dUTP	2 µl	40 µM
DNA template	variable (1 µg)	0.02 µg/µl
DNase I (1000 times diluted from a 1 mg/ml stock)	1 µl	0.02 ng/ml
DNA Polymerase (10 U)	1 µl	0.2 U/µl
H_2O	to 50 µl	—
Total Volume	**50 µl**	

❷ Mix and incubate the reaction at +15°C for 30–120 min.

❸ Use an aliquot to verify the probe size by gel electrophoresis.

❹ Add 5 µl 500 mM EDTA to the reaction tube.
This terminates the labeling reaction.

The fragment length of the nick-translated DIG probe can be manipulated by monitoring the time of the reaction or by varying the DNase concentration. For *in situ* hybridization procedures, the optimal fragment length is about 200–400 bases. For the analysis of fragment length by gel electrophoresis, see Sambrook *et al.* (3) If the fragment length is too large, the labeled probe can be sonicated.

WHAT TO DO NEXT *For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.*

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for in situ hybridization, see page 38.

References

1. Rigby, P.W.J., Dieckmann, M., Rhodes, C., Berg, P. (1977) Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**:237–241.
2. Langer, P.R., Waldrop, A.A., Ward, D.C. (1981) Enzymatic synthesis of biotin-labeled polynucleotides: Novel nucleic acid affinity probes. *Proc. Natl. Acad. Sci. USA* **78**(11):6633–6637.
3. Sambrook, J., Fritsch, E.M. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Preparing cDNA with Digoxigenin-11-dUTP

Using AMV Reverse Transcriptase, you can generate DIG-labeled cDNA. The following reaction conditions will produce cDNA probes with optimal signal intensity. Lower levels of Digoxigenin-11-dUTP result in decreased signal intensity. Greater amounts of DIG-11-dUTP may yield cDNA probes that result in increased background.

Standard Labeling Reaction

Products required

While the cDNA Synthesis Kit will synthesize cDNA from RNA, it is not optimized for the incorporation of digoxigenin-11-dUTP (DIG-11-dUTP); therefore, the required products should be purchased separately.

Name in procedure	Description	Available as
RNase inhibitor	25 units/µl RNase inhibitor from human placenta	Cat. No. 799 017
dNTP mixture	dATP, dCTP, dGTP; 5 mM each	dATP (Cat. No. 1051 440); dCTP (Cat. No. 1051 458); dGTP (Cat. No. 1051 466); (sold as a 100 mM stock solutions; must be diluted before use)
dTTP	0.65 mM dTTP	Cat. No. 1051 482 (sold as a 100 mM stock solution; must be diluted before use)
DIG-dUTP	0.35 mM digoxigenin-11-dUTP (2'-deoxy-uridine-5-triphosphate coupled to digoxigenin via an 11-atom spacer arm) in redistilled water	1. alkali-labile, Cat. Nos. 1573 152, 1573 179 2. alkali-stable, Cat. Nos. 1093 088, 1558 706, 1570 013 (both sold as a 1 mM solution; must be diluted before use)
Oligo-p$(dT)_{15}$	0.74 µg/µl oligo$(dT)_{15}$ primer	Cat. No. 814 270
AMV Reverse Transcriptase	25 units/µl reverse transcriptase from Avian Myelobastosis Virus (AMV)	Cat. No. 109 118

Additionally required solutions

In addition to your RNA template, you will need the following reagents and solutions.

Additionally required solutions	Description
5X cDNA reaction buffer	250 mM Tris-HCl, pH 8.5; 40 mM $MgCl_2$; 150 mM Dithiothreitol (DTT)
DEPC-treated H_2O	Sterile, distilled water treated with 0.1% diethylpyrocarbonate (DEPC)
EDTA	200 mM EDTA, pH 8.0

Procedure

❶ Heat-denature the RNA template in a boiling water bath for 10 minutes, and chill it on dry ice/Ethanol for 30 seconds before use.

❷ Add reagents to a sterile microfuge tube (on ice) in the following order:

Reagent	Volume	Final Concentration
5X cDNA reaction buffer	6 µl	1X
RNase inhibitor	1.5 µl	1.25 units/µl
dNTP mixture	3 µl	0.5 mM each
dTTP	6 µl	0.13 mM
DIG-dUTP	6 µl	0.07 mM
Oligo-p$(dT)_{15}$	3 µl	0.074 µg/µl
heat-denatured RNA	variable (1.5 µg)	0.05 µg/µl
DEPC-treated H_2O	to 28 µl	——
AMV Reverse Transcriptase	2 µl	1.67 units/µl
Total Volume	**30 µl**	

❸ Incubate the reaction at +42°C for 90 minutes.

❹ Add 1 µl (200 mM) EDTA to the reaction tube.
This terminates the labeling reaction.

WHAT TO DO NEXT *For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.*

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for in situ hybridization, see page 38.

Chapter 3 *Oligonucleotide Labeling*

A Comparison of Oligonucleotide-labeling Methods

Synthetic oligonucleotide probes are widely used in library-screening procedures, Southern and northern blots, dot blots, and *in situ* hybridization experiments. To provide researchers with maximum flexibility, Boehringer Mannheim has developed three methods for labeling oligonucleotides with digoxigenin (Figure 5).

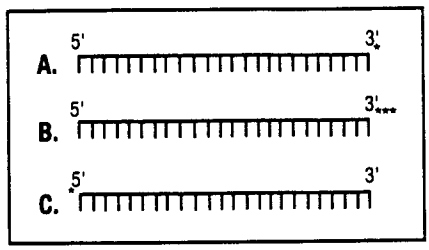

Figure 5. Options for nonradioactive oligonucleotide labeling. An asterisk (*) indicates placement of a single digoxigenin residue. A. 3'-End labeling; B. 3' Tailing; C. 5'-End labeling.

This section briefly outlines the three oligonucleotide-labeling methods, each of which produces probes that are optimized for specific applications (see Table 4).

Labeling method	Amount of oligo produced	Probe sensitivity	Probe specificity	Major characteristics	Applications
3'-end labeling	100 pmol per reaction	++	+++	—Addition of a single DIG residue	*In situ* hybridization Northern blots Southern blots Colony/plaque hybridization Dot/slot blotting
3' tailing	100 pmol per reaction	+++	++	—Addition of multiple DIG residues	Northern blots *In situ* hybridization Sequencing Southern blots Colony/plaque hybridization
5'-end labeling with DIG-NHS ester	100 nmol per reaction	++	+++	—Oligo must be synthesized with aminolinker —Good for large-scale labeling —Chemical reaction	Primer extension Sequencing Dot/slot blots Colony/plaque hybridization *In situ* hybridization

Table 4. Overview of oligonucleotide-labeling methods

Oligonucleotide 3'-End Labeling (Genius 5 Kit)

The **Genius 5 Oligonucleotide 3'-End Labeling Kit (Cat. No. 1362 372)** is designed for the addition of digoxigenin-11-ddUTP (DIG-ddUTP) to the 3' end of a synthetic oligonucleotide 14–100 nucleotides in length. The enzyme terminal transferase adds one digoxigenin residue per oligonucleotide because chain elongation cannot proceed past the dideoxy nucleotide. Each labeling reaction generates about 100 pmol of labeled probe (equal to 1 µg of a 30-mer). Probes labeled with this method retain their high degree of specificity and, despite the additional dideoxynucleotide, can still be treated under the same optimal hybridization and washing conditions (*i.e.*, temperature and salt concentration). In addition, this method enables nonradioactive DIG labeling of conventionally synthesized oligonucleotides; therefore, the nonradioactive label DIG-ddUTP can be linked to the oligonucleotide without using any special reagents for oligonucleotide synthesis. These probes are particularly suited for experiments that require maximum probe specificity and moderate probe sensitivity (see Table 4).

Oligonucleotide 3' Tailing (Genius 6 Kit)

The **Genius 6 Oligonucleotide Tailing Kit (Cat. No. 1417 231)** is designed for the addition of a tail of residues ranging from 10–100 bases in length. In the 3' tailing reaction, terminal transferase adds a mixture of unlabeled nucleotides and digoxigenin-11-dUTP, producing a tail containing multiple digoxigenin residues. The resulting probes are about ten times more sensitive than 3'-end-labeled probes produced with the Genius 5 Kit.

Although tailed oligonucleotide probes are more sensitive than 3'-end-labeled probes, they can also produce non-specific background due to the presence of the longer tail. For example, if the unlabeled nucleotide used in the tailing reaction is dATP, the probe may be inclined to anneal to T-rich regions in complex nucleic acid mixtures. Such non-specific signals can be minimized by choosing a different unlabeled nucleotide to utilize in the tailing reaction, by prehybridizing with a competing sequence [*e.g.*, poly(A)], or by altering the stringency conditions. Tailed probes are suitable for procedures requiring optimal sensitivity, such as Southern and northern blots.

5'-End Labeling Oligonucleotides (Genius 8 Set)

Oligonucleotides can be chemically tagged with digoxigenin at the 5' end in a two-step procedure with the **Genius 8 Oligonucleotide 5'-End Labeling Set (Cat. No. 1480 863)**. In the first step, the oligonucleotide is synthesized with an aminolinker residue on its 5' end. After the synthetic oligonucleotide is purified, the second step involves the covalent linkage of digoxigenin-NHS ester to the free 5'-amino residue.

The resulting probes can be produced in large quantities (100 nmol per reaction), are specific, and have a sensitivity comparable to that of 3'-end-labeled probes (*i.e.*, approximately 10 pg can be detected in a dot blot). The probes are suitable for applications such as library screening and dot blot hybridization experiments.

Another useful feature of 5'-end-labeled oligonucleotides is that the 3' end is free to act as a primer for DNA-synthesis reactions. Thus, extension reactions, such as PCR, can be conducted with labeled primers, allowing the nonradioactive tagging of the reaction products. Subsequently, the labeled extension products can be detected or be purified by affinity chromatography using anti-digoxigenin antibodies.

WHAT TO DO NEXT *At this time, proceed to the section on the appropriate oligonucleotide-labeling method to prepare your probe.*

3'-End Labeling Oligonucleotides with Digoxigenin-11-ddUTP

Standard 3'-End Labeling Reaction

Products required

The **Genius 5 Oligonucleotide 3'-End Labeling Kit (Cat. No. 1362 372)** contains all of the components needed to make 3'-end-labeled oligonucleotide probes with digoxigenin. The kit also contains a DIG-ddUTP-labeled Control Oligonucleotide, which should be used in a direct detection assay to estimate the yield of DIG-labeled oligonucleotide (see page 33), as well as an Unlabeled Control Oligonucleotide for labeling, and a Control DNA for hybridization. The DIG-ddUTP-labeled Control Oligonucleotide and kit components required for labeling are also available as separate products.

Name in procedure	Description	Available as
5X reaction buffer	1 M potassium cacodylate*, 125 mM Tris-HCl, 1.25 mg/ml bovine serum albumin; pH 6.6 (+25°C)	1. Vial 1, Genius 5 Kit 2. Cat. No. 1243 276 3. (supplied with terminal transferase)
$CoCl_2$ solution	25 mM cobalt chloride ($CoCl_2$)	1. Vial 2, Genius 5 Kit 2. Cat. No. 1243 306 3. (supplied with terminal transferase)
DIG-ddUTP	1 mM digoxigenin-11-ddUTP (2',3'-dideoxyuridine-5'-triphosphate, coupled to digoxigenin via an 11-atom spacer arm) in redistilled water	1. Vial 3, Genius 5 Kit 2. Cat. No. 1363 905
Terminal transferase	50 units/µl terminal transferase, in 200 mM potassium cacodylate*, 1 mM EDTA, 200 mM KCl, 0.2 mg/ml bovine serum albumin; 50% (v/v) glycerol; pH 6.5 (+25°C)	1. Vial 4, Genius 5 Kit 2. Cat. No. 220 582 (sold at 25 U/µl; contains 5X reaction buffer and cobalt chloride)
Unlabeled Control Oligonucleotide (for control reactions only)	30-mer, 5'-pTTG GGT AAC GCC AGG GTT TTC CCA GTC ACG OH-3', homologous to the *lac* Z' region in pUC and M13 plasmids, in redistilled water	Vial 5, Genius 5 Kit

*Potassium cacodylate is toxic. Wear gloves when handling. Discard as regulated for toxic waste.

Additionally required solutions

In addition to the Genius 5 Kit and your oligonucleotide, you will need the following solutions.

Additionally required solutions	Description
H_2O	Sterile, distilled water
EDTA	200 mM EDTA, pH 8.0

Procedure

Purify (by HPLC or gel electrophoresis) the oligonucleotide to be labeled after synthesis. Most suppliers will do this for the customer.

❶ Add reagents to a sterile microfuge tube (on ice) in the following order:

Reagents	Volume	Volume (Control Reaction)	Final Concentration
5X reaction buffer	4 µl	4 µl	1X
$CoCl_2$ solution	4 µl	4 µl	5 mM
oligonucleotide	variable (100 pmol)	—	5 pmol/µl
OR			
Unlabeled Control Oligonucleotide	—	5 µl (100 pmol)	5 pmol/µl
DIG-ddUTP	1 µl	1 µl	0.05 mM
Terminal transferase	1 µl	1 µl	2.5 units/µl
H_2O	to 20 µl	5 µl	—
Total Volume	**20 µl**	**20 µl**	

❷ Incubate the reaction at +37°C for 15 minutes. Place on ice.

❸ Add 1 µl EDTA to the reaction tube.
This terminates the labeling reaction.

Notes on subsequent hybridization

The labeled probe may be diluted in hybridization buffer without ethanol precipitation. The presence of unincorporated DIG-ddUTP will not cause a background problem if SSC buffer is used for hybridization and wash steps. However, if TMAC is used in the wash and hybridization buffer, ethanol precipitate the labeled probe if it is to be used in *in situ* hybridization (see page 38).

WHAT TO DO NEXT *For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.*

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for **in situ** *hybridization, see page 38.*

3' Tailing Oligonucleotides with Digoxigenin-11-dUTP/dATP

Standard Tailing Reaction

Products required

The **Genius 6 Oligonucleotide Tailing Kit (Cat. No. 1417 231)** contains all of the components needed to make tailed probes containing DIG-dUTP/dATP residues. Nucleotides other than the dATP may be used to tail oligonucleotides; see page 26 for the modified procedure. The kit also contains a DIG-dUTP/dATP-tailed Control Oligonucleotide, which should be used in a direct detection assay to estimate the yield of DIG-labeled oligonucleotide (see page 33). In addition, the kit contains an Unlabeled Control Oligonucleotide for labeling and a Control DNA for hybridization. The kit components required for tailing and DIG-dUTP/dATP-tailed Control Oligonucleotide are also available as separate items.

Name in procedures	Description	Available as
5X reaction buffer	1 M potassium cacodylate*, 125 mM Tris-HCl, 1.25 mg/ml bovine serum albumin; pH 6.6 (+25°C)	1. Vial 1, Genius 6 Kit 2. Cat. No. 1243 276 3. (supplied with terminal transferase)
$CoCl_2$ solution	25 mM cobalt chloride ($CoCl_2$)	1. Vial 2, Genius 6 Kit 2. Cat. No. 1243 306 3. (supplied with terminal transferase)
DIG-dUTP	1 mM digoxigenin-11-dUTP (2'-deoxyuridine-5'-triphosphate, coupled to digoxigenin via an 11-atom spacer arm) in redistilled water	1. Vial 3, Genius 6 Kit 2. alkali-labile, Cat. Nos. 1573 152, 1573 179 3. alkali-stable, Cat. Nos. 1093 088, 1558 706, 1570 013
dATP	10 mM dATP solution; in Tris buffer, pH 7.5	1. Vial 4, Genius 6 Kit 2. Cat. No. 1051 440 (sold as a 100 mM solution; must be diluted before use)
Terminal transferase	50 units/µl terminal transferase, in 200 mM potassium cacodylate*, 1 mM EDTA, 200 mM KCl, 0.2 mg/ml bovine serum albumin; 50% (v/v) glycerol; pH 6.5 (+25°C)	1. Vial 5, Genius 6 Kit 2. Cat. No. 220 582 (sold at 25 U/µl; contains 5X reaction buffer and cobalt chloride)
Unlabeled Control Oligonucleotide (for control reactions only)	30-mer, 5'-pTTG GGT AAC GCC AGG GTT TTC CCA GTC ACG OH-3', homologous to the *lac* Z' region in pUC and M13 plasmids, in redistilled water	Vial 6, Genius 6 Kit

*Potassium cacodylate is toxic. Wear gloves when handling. Discard as regulated for toxic waste.

Additionally required solutions

In addition to the Genius 6 Kit and your oligonucleotide, you will need the following solutions.

Additional solutions	Description
H_2O	Sterile, distilled water
EDTA	200 mM EDTA, pH 8.0

Procedure

The oligonucleotide to be labeled should be purified by HPLC or gel electrophoresis after synthesis. Most suppliers will do this for the customer.

❶ Add reagents to a sterile microfuge tube (on ice) in the following order:

Reagents	Volume	Volume (Control Reaction)	Final Concentration
5X reaction buffer	4 µl	4 µl	1X
$CoCl_2$ solution	4 µl	4 µl	5 mM
DIG-dUTP	1 µl	1 µl	0.05 mM
oligonucleotide	variable (100 pmol)	—	5 pmol/µl
OR			
Unlabeled Control Oligonucleotide	—	5 µl (100 pmol)	5 pmol/µl
dATP	1 µl	1 µl	0.5 mM
terminal transferase	1 µl	1 µl	2.5 units/µl
H_2O	to 20 µl	4 µl	—
Total Volume	**20 µl**	**20 µl**	

Do not increase the size of the labeling reaction beyond 40 µl because this will result in inefficient labeling of the oligonucleotide.

❷ Incubate the reaction at +37°C for 15 minutes. Quickly place on ice.

❸ Add 1 µl EDTA to the reaction tube.
This terminates the labeling reaction.

Notes on subsequent hybridization

- We have occasionally experienced background problems when probes labeled in this manner are used in hybridization buffers containing TMAC. Hybridization buffers containing SSC are preferable when "long-tailed" probes are used. If it is necessary to use TMAC, we recommend the use of an oligonucleotide probe labeled with DIG-ddUTP as well as ethanol precipitation before use.
- Use of tailed probes in hybridization experiments can sometimes cause nonspecific hybridization of the tail to complementary sequences in the target DNA. To prevent this, add 0.1 mg/ml poly(A) [vial 11 or Cat. No. 108 626] and/or 5 µg/ml poly(dA) [Cat. No. 223 581] to the hybridization buffer; this will block the target sequences.
- Both the optimal concentration of labeled probe in the hybridization buffer and the time required for hybridization depend on the amount of DNA or RNA that will be detected on a filter. Usually, the probe is diluted to 1–10 pmol/ml, and the hybridization is carried out for 1–6 hours in 10 ml of hybridization buffer per 100 cm^2 of membrane.
- Heat-denature the oligonucleotide prior to hybridization if secondary structure can be expected from the oligonucleotide sequence.

WHAT TO DO NEXT *For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.*

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for in situ hybridization, see page 38.

Oligonucleotide Tailing with Nucleotides Other than dATP

Oligonucleotides can also be tailed with DIG-dUTP and dGTP, or dCTP and dTTP, or a mixture of all four unlabeled deoxynucleoside triphosphates. See Table 5 for tail lengths and incorporation rates for other nucleotides.

DIG-dUTP/dNTP labeling mixture,1:10	dATP	dCTP	dGTP	dTTP	dNTP
Average tail length	50	25	15	10	5
Range of tail length	10–100	10–40	10–25	1–20	1–10
DIG-dUTP/tail	5	2.5	1.5	1	0.5

Table 5. Tail-lengths and incorporation rates for other nucleotides.

Products required

In addition to the **Genius 6 Oligonucleotide Tailing Kit (Cat. No. 1417 231)**, the following products can be purchased separately when tailing oligonucleotides with other nucleotides.

[illegible]	Description	Availability
dCTP	100 mM dCTP	Cat. No. 1051 458
dGTP	100 mM dGTP	Cat. No. 1051 466
dTTP	100 mM dTTP	Cat. No. 1051 482
deoxynucleotide triphosphate set	dATP, dCTP, dGTP, dTTP; 100 mM each; pH 7.0	Cat. No. 1277 049

Procedure

❶ Mix 9 volumes of DIG-dUTP with 1 volume of the appropriate deoxynucleotide triphosphate solution (to be chosen from the "Available reagent" list [above]).

This DIG-dUTP/dNTP tailing mixture will be added to the oligonucleotide tailing reaction in place of the DIG-dUTP and dATP.

❷ Add reagents to a sterile microfuge tube (on ice) in the following order:

Reagents	Volume	Volume (Control Reaction)	Final Concentration
5X reaction buffer	4 µl	4 µl	1X
$CoCl_2$ solution	4 µl	4 µl	5 mM
DIG-dUTP/dNTP tailing mixture	1 µl	1 µl	0.5/0.5 mM
oligonucleotide **OR**	variable (100 pmol)	—	5 pmol/µl
Unlabeled Control Oligonucleotide	—	5 µl (100 pmol)	5 pmol/µl
terminal transferase	1 µl	1 µl	2.5 units/µl
H_2O	to 20 µl	5 µl	——
Total Volume	**20 µl**	**20 µl**	

❸ Incubate the reaction at +37°C for 15 minutes. Place on ice.

❹ Add 1 µl EDTA to the reaction tube.

This terminates the labeling reaction.

WHAT TO DO NEXT *For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.*

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for in situ hybridization, see page 38.

5'-End Labeling Oligonucleotides with Digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxy-succinimide Ester (DIG-NHS ester)

Standard 5'-End Labeling Reaction

With the **Genius 8 Oligonucleotide 5'-End Labeling Set (Cat. No. 1480 863)**, oligonucleotides can be labeled with digoxigenin at the 5' end after synthesis that includes the addition of a phosphoramidite. First, oligonucleotides are reacted with the phosphoramidite in a final synthesis step according to the solid-phase phosphoramidite method, creating a 5'-terminal amino function. Treatment with ammonia releases the oligonucleotide from the support and cleaves the protecting groups. In the subsequent step, digoxigenin is introduced at the 5' end.

Products required

[illegible]	Description	Available
Aminolinker*	[N-Trifluoroacetamido-(3-oxa)-pentyl-N,N-diisopropyl-methyl]-phosphoramidite	Vial 1, Genius 8 Set
DIG-NHS ester†	Digoxigenin-3-O-methylcarbonyl-ε-amino-caproic acid-N-hydroxysuccinimide ester	1. Vial 2, Genius 8 Set 2. Cat. No. 1333 054

* The aminolinker reacts violently with water, and it is irritating to eyes, respiratory system, and skin.
† DIG-NHS ester is very toxic by inhalation, in contact with skin, or swallowed. Do not breath dust.

Additionally required solutions

In addition to the Genius 8 Set, you will need the following solutions.

[illegible]	Description
Acetonitrile	anhydrous acetonitrile
Aqueous ammonia	25% aqueous ammonium
H_2O	Sterile, distilled water
Sodium acetate	3 M sodium acetate, pH 8.5
Ethanol	Absolute ethanol, chilled at –20°C; when 70% ethanol is indicated, dilute ethanol with redistilled water.
Sodium borate	100 mM sodium borate; pH 8.5
Elution buffer A	100 mM triethylammonium acetate, pH 6.8
Elution buffer B	100 mM triethylammonium acetate:acetonitrile (1:1), pH 6.8

Introduction of the 5' amino function

The Aminolinker used corresponds to the phosphoramidites used in oligonucleotide synthesis protocol (1).

The bottle with the Aminolinker fits directly into the appropriate position of an automatic DNA synthesizer from Applied Biosystems, Pharmacia, or Eppendorf.

For use in the synthesizers from Milligen/Biosearch, the bottle content has to be dissolved in the appropriate amount of anhydrous acetonitrile (see below) by injection of the solvent into the sealed bottle with a disposable syringe and subsequent transfer to the reservoir at the synthesizer.

1. Dissolve 100 mg Aminolinker in Anhydrous Acetonitrile for the synthesizers from Applied Biosystems, Pharmacia, Eppendorf in 2.7 ml (100 mM); Milligen/Biosearch in 4.3 ml (70 mM).
   Note: The solution of the phosphoramidite is stable for approx. 2 weeks at ambient temperature and exclusion of moisture.
2. Start oligonucleotide synthesis according to standard protocol. Set the synthesizer on "trityl on."
3. Deprotect the oligonucleotide according to standard oligonucleotide synthesis (by treatment with 25% aqueous ammonia).
4. Remove ammonia by evaporation or lyophilization.

Ethanol precipitation of the oligonucleotide

❶ Dissolve the oligomer (approx. 100 nmol) in a mixture of 300 µl H_2O and 30 µl 3 M Sodium acetate, pH 8.5, 1 µl glycogen, and transfer into an microfuge tube.

❷ Add 0.9 ml ice-cold Ethanol. Mix well.

❸ Incubate at –20°C for 2–3 h.

❹ Centrifuge for 15 min at 10,000 x *g*. Decant the supernatant.

❺ Wash the pellet with 100 µl of ice-cold Ethanol, centrifuge for 5 min at 10,000 x *g*, and decant the supernatant.

Labeling reaction (example of a 20-mer)

❶ Dissolve the oligonucleotide pellet (approx. 20 A_{260} units, corresponding to approx. 100 nmol) in 200 µl Sodium borate.

❷ Dissolve the content of a vial (1 mg) of the DIG-NHS-ester in 600 µl Ethanol, and add 200 µl of this solution to the oligonucleotide solution.

Note: The solution of the DIG-NHS ester in ethanol is only stable for a short time. Therefore, we recommend that you use the ester solution for multiple parallel labelings (***e.g.***, in this case, for two additional reactions).

In general, 1 mg (1.5 µmol) of the DIG-NHS ester is sufficient for labeling 300 nmol of 5'-amino-substituted oligonucleotide. Starting with 20 A_{260} units of oligomer, 1 mg DIG-NHS ester is sufficient for

- 2 labeling reactions of a 15-mer
- 3 labeling reactions of a 20-mer
- 4 labeling reactions of a 25-mer.

❸ Place vials on a shaker platform overnight at room temperature.

Purification of the labeled oligonucleotide

Separation of the labeled oligonucleotide from the unlabeled compound may be achieved by reversed phase HPLC*.

❶ Concentrate the mixture of the labeling reaction under vacuum.

❷ Dissolve the remainder in 1 ml of H_2O.

❸ Pass this mixture through a 0.45 µm filter.

❹ Apply onto a HPLC column, RP-18/5 µm.

Gradient: In 30 min from 100% Elution buffer A to 80% Elution buffer B.

The digoxigenin-labeled oligonucleotide is eluted with a higher retention value compared to the unlabeled compound. A typical elution profile is shown in Figure 6. An average yield of 50% digoxigenin-labeled oligomers is obtained.

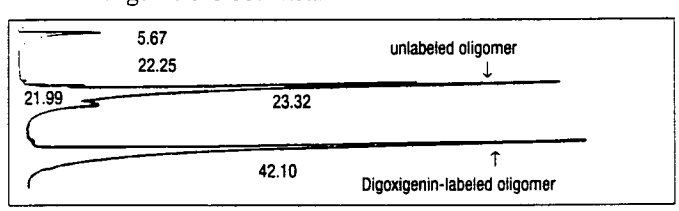

Figure 6. Elution profile. Oligonucleotides were purified after labeling with DIG-NHS Ester by reversed phase HPLC with an Inertsil™ column. Columns from other manufacturers give similar elution profiles, though the distance between the peaks can vary.
A. unlabeled oligonucleotide
B. DIG-labeled oligonucleotide

❺ Concentrate the appropriate fraction under vacuum. Desalt as usual (e.g., gel filtration, dialysis in SPECTRAPOR® 1000).

*Alternatively, the separation from unlabeled oligonucleotides can be achieved by standard polyacrylamide gel electrophoresis. As an additional alternative, the labeled oligonucleotide may be purified on RP-C-18 cartridges (*e.g.*, Poly-Pak RP1 from MWG Biotech, Roth; OPC from ABI).

WHAT TO DO NEXT *For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.*

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Labeling RNA with the Genius 4 Kit

The Genius 4 Kit generates DIG-labeled, single-stranded RNA probes of defined length by transcription. RNA probes are labeled with digoxigenin, using SP6, T7, or T3 RNA polymerases. DNA is subcloned into a multiple cloning site adjacent to the RNA polymerase promoter site in the pSPT18 or pSPT19 transcription vectors provided in the kit. A restriction enzyme (not provided in the kit) linearizes the DNA template, allowing the creation of "run off" transcripts of uniform length. One digoxigenin-11-UTP residue is incorporated every 20–25 nucleotides.

A large amount of DIG-labeled RNA can be generated by this method because the nucleotide concentration does not become limiting in the standard transcription assay. Under standard conditions, approximately 2–10 µg of full-length DIG-labeled RNA is transcribed from 1 µg of template DNA.

The RNA probes produced with this method are desirable because they:

- have a defined unit length
- exhibit single-strand target specificity
- do not re-anneal like double-stranded DNA probes.

RNA labeled with digoxigenin is particularly useful for northern blots. In addition, DIG-labeled RNA probes can be used for Southern blots, plaque or colony screening, and *in situ* hybridization without removing the label. Also, because the linkage between DIG and UTP is resistant to alkali, DIG-labeled RNA can be fragmented by alkaline treatment. When creating RNA probes for *in situ* hybridization, a limited reduction in size of the DIG-labeled RNA probe can be advantageous (see the section entitled "Regulation of RNA Probe Length by Alkaline Hydrolysis" on page 32 for details).

Standard Labeling Reaction

Products required

The most convenient approach to making RNA probes is to use the **Genius 4 RNA Labeling Kit (Cat. No. 1175 025)** because it contains most of the components needed to make RNA probes. The kit also contains a number of control RNAs and DNAs, which can be used in hybridizations, direct detections, or to check the efficiency of the labeling reaction (See page 33). Some of the kit components are also available as separate products, but the buffers and some of the controls are not.

	Description	Availability
NTP labeling mixture (10X)	10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-UTP; in Tris-HCl, pH 7.5 (+20°C)	1. Vial 7, Genius 4 Kit 2. Cat. No. 1277 073
10X transcription buffer	400 mM Tris-HCl, pH 8.0; 60 mM $MgCl_2$, 100 mM dithioerythritol (DTE), 20 mM spermidine, 100 mM NaCl, 1 unit/ml RNase inhibitor	Vial 8, Genius 4 Kit
DNase I, RNase-free	10 units/µl DNase I, RNase-free	1. Vial 9, Genius 4 Kit 2. Cat. No. 776 785
RNase inhibitor	20 units/µl RNase inhibitor	1. Vial 10, Genius 4 Kit 2. Cat. No. 799 017
One of the following		
SP6 RNA Polymerase	20 units/µl SP6 RNA Polymerase	1. Vial 11, Genius 4 Kit 2. Cat. No. 810 266
T7 RNA Polymerase	20 units/µl T7 RNA Polymerase	1. Vial 12, Genius 4 Kit 2. Cat. No. 881 767
T3 RNA Polymerase	20 units/µl T3 RNA Polymerase	Cat. No. 1031 163
One of the following (for control reactions only):		
Control DNA 1, pSPT-18 Neo	0.25 mg/ml pSPT-18 Neo DNA, cleaved with *Pvu* II	Vial 3, Genius 4 Kit
OR		
Control DNA 2, pSPT-19 Neo	0.25 mg/ml pSPT-19 Neo DNA, cleaved with *Pvu* II	Vial 4, Genius 4 Kit

Additionally required solutions

In addition to the Genius 4 Kit and your purified DNA template, you will need the following solutions.

Additionally required solutions	Description
DEPC-treated H_2O	Sterile, distilled water treated with 0.1% diethylpyrocarbonate (DEPC)
EDTA	200 mM EDTA, pH 8.0

Procedure

Before beginning the transcription reaction, the DNA template must be linearized at a restriction enzyme site downstream of the cloned insert. To avoid transcription of undesirable sequences, use a restriction enzyme that leaves 5' overhangs or blunt ends. After the restriction digest, purify the DNA by phenol/chloroform extraction, followed by ethanol precipitation (see page 38). Alternatively, we have found Elutip™-r columns (Schleicher & Schuell; Cat. No. NA 020/2) to be an easy and effective method of purifying DNA template without contaminating the sample with RNase.

❶ Add reagents to a sterile, RNase-free (DEPC-treated) microfuge tube (on ice) in the following order:

Reagents	Volume	Volume (Control Reaction)	Final Concentration
Purified DNA template **OR**	variable (1 µg)	—	0.05 µg/µl
Control DNA 1 or 2	—	4 µl	0.05 µg/µl
NTP labeling mixture (10X)	2 µl	2 µl	1X
10X transcription buffer*	2 µl	2 µl	1X
DEPC-treated H_2O	to 18 µl	10 µl	——
RNA polymerase (SP6, T7, or T3)	2 µl	2 µl	2 units/µl
Total Volume	**20 µl**	**20 µl**	

*Optional: Add an additional 1 µl of RNase inhibitor.

The reaction may be scaled up to increase the yield of RNA. This is achieved by keeping the amount of template DNA constant while increasing the amount of the other components in the labeling reaction. For example, in a 5X scaled-up reaction with 1 µg of linear Control DNA 1 (pSPT18-Neo DNA) as template, more than 40 µg of RNA can be synthesized after a two-hour incubation at +37°C.

❷ Mix gently and centrifuge briefly. Incubate for at least 2 hours at +37°C.

❸ If desired, add 2 µl DNase I, RNase-free (Cat. No. 776 785), and incubate for 15 minutes at +37°C to remove the DNA template.

Because the amount of DIG-labeled RNA transcript greatly exceeds the amount of DNA template, removal of the DNA template is usually unnecessary.

❹ With or without prior DNase treatment, add 2 µl EDTA solution to the reaction tube.

This terminates the transcription reaction.

The amount of newly synthesized DIG-labeled RNA depends on the amount, size (site of linearization), and purity of the template DNA. When 1 µg of template DNA that has been linearized to give run off transcripts of 760 bases is labeled in the standard reaction, approximately 37% of the nucleotides are incorporated into about 10 µg of transcribed DIG-labeled RNA.

The RNA transcripts can be analyzed for size by agarose gel electrophoresis (*e.g.*, formaldehyde gels) and ethidium bromide staining. Labeling efficiency can be most accurately checked by direct detection of the labeled RNA probe with Anti-digoxigenin-alkaline phosphatase.

WHAT TO DO NEXT *For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.*

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for in situ hybridization, see page 38.

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Regulation of RNA Probe Length by Alkaline Hydrolysis

Some applications require shorter RNA probes than other techniques. When performing *in situ* hybridizations, for example, probes must be short enough to allow diffusion into and out of the tissue. Alkaline hydrolysis allows you to regulate the size of RNA probes.

Products required

Name in procedure	Description	Available as
Glycogen solution	20 mg/ml glycogen in redistilled water	Cat. No. 901 393

Additionally required solutions

Additionally required solutions	Description
DEPC-treated H_2O	Sterile, distilled water treated with diethylpyrocarbonate (DEPC)
Carbonate buffer	60 mM Na_2CO_3; 40 mM $NaHCO_3$; pH 10.2
Hydrolysis-neutralization buffer	200 mM sodium acetate; 1% (v/v) acetic acid; pH 6.0
Ethanol	Absolute ethanol, chilled at –20°C; when 70% ethanol is indicated, dilute the ethanol with DEPC-treated water.

Procedure

The following procedure is a modification of the protocol for regulating the size of RNA probes by alkaline hydrolysis described by Cox, *et al.* (1984, *Develop. Biol.* **101**:485–502.). This protocol was adapted for use with DIG-UTP-labeled RNA probes.

1. Hydrolyze 1 µg RNA by adding an equal volume of DEPC-treated H_2O and two volumes of Carbonate buffer. Incubate for 10–60 minutes at +60°C.

   The optimal incubation time must be determined empirically. We have found that hydrolysis starts as early as 30 seconds after the addition of the carbonate buffer.

2. Add an equal volume of Hydrolysis-neutralization buffer to stop the hydrolysis.
3. Add 1 µl Glycogen solution and 3 volumes of chilled Ethanol to precipitate the RNA. Mix well and incubate at –70°C for 30 minutes.
4. Remove from the –70°C incubation, and thaw briefly at room temperature. Centrifuge at 13,000 x *g* for 15 minutes in a microcentrifuge.
5. Decant the Ethanol, and wash the pellet with 100 µl of 70% ethanol. Centrifuge at 13,000 x *g* for 5 minutes in the microcentrifuge, then remove the 70% ethanol.
6. Dry the pellet and resuspend in 100 µl DEPC-treated H_2O. If not used immediately, store the probe at –70°C.

   Note: Resuspension of the digoxigenin-labeled pellet may require heating to +37°C for 10 minutes with frequent vortexing. Failure to adhere to these guidelines will result in the loss of approximately 20% of the digoxigenin-labeled RNA.
7. Remove 2 µl RNA and dilute in 98 µl DEPC-treated H_2O. Check the resulting probe's length by electrophoresis of 4 µl of diluted, hydrolyzed RNA on a 1% denaturing agarose gel (glyoxal gel works best), followed by transfer to a nylon membrane. Visualize the labeled RNA probe by direct immunodetection according to steps 6–13 in "Estimating the Yield of DIG-labeled Nucleic Acids" on page 33.

WHAT TO DO NEXT *For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33 and perform steps 6–13.*

Chapter 5 *Estimating the Yield of DIG-Labeled Nucleic Acids*

It is essential that you estimate the yield of each labeling reaction before attempting to use the probe in a hybridization reaction. Use the following procedure to estimate the yield of DIG-labeled DNA, RNA, or oligonucleotide probes. The procedure described here will

- confirm that the labeling reaction has been successful in producing a DIG-labeled probe
- estimate the yield of DIG-labeled probe
- familiarize the user with the basic detection methodology of the Genius System.

The detection procedure described here should only be used to estimate the yield of DIG-labeled nucleic acids and confirm the success of the labeling reaction. More detailed and sensitive detection procedures for blotting applications (*e.g.*, Southern blots) are detailed in the "**Hybridization**" division, which begins on page 41.

Products required

DIG-labeled controls for estimating the yield of DNA, RNA, and end-labeled oligonucleotide probes are available as separate reagents or in the respective labeling kits. The DIG-dUTP/dATP-tailed Oligonucleotide Control is only available in the Genius 6 Kit.

Use Table 6 to determine the reagents and dilution scheme required to estimate the yield of your probe. Choose the control nucleic acid corresponding to the type of probe you used, and perform the procedure provided below.

If you labeled your probe by:	Use:	Perform dilutions with:	Perform dilution scheme:
PCR	Labeled Control DNA	DNA dilution buffer	A
Random primed labeling	Labeled Control DNA	DNA dilution buffer	A
Nick translation	Labeled Control DNA	DNA dilution buffer	A
Preparing cDNA with DIG-dUTP	Labeled Control DNA	DNA dilution buffer	A
Oligonucleotide 3'-end labeling	Control Oligonucleotide, DIG-ddUTP-labeled	DNA dilution buffer	B
Oligonucleotide tailing	Control Oligonucleotide, DIG-dUTP/dATP-tailed	DNA dilution buffer	B
Oligonucleotide 5'-end labeling	Control Oligonucleotide, DIG-ddUTP-labeled	DNA dilution buffer	B
RNA labeling (transcription)	Labeled Control RNA	DEPC-treated H_2O	C

Table 6. Procedural differences for estimating the yield of different probe-labeling reactions.

This procedure requires only one of the following controls.

DIG-labeled control (choose one):	Description:	Available in:
Labeled Control DNA	Digoxigenin-labeled pBR328 DNA that has been random primed labeled according to the standard labeling procedure; the total DNA concentration in the vial is 20 µg/ml, but only 5 µg/ml of it is DIG-labeled DNA.	1. Vial 4, Genius 1 Kit 2. Vial 4, Genius 2 Kit 3. Vial 1, Genius 3 Kit 4. Cat. No. 1585 738
Control Oligonucleotide, DIG-ddUTP-labeled	2.5 pmol/µl oligonucleotide (30-mer, 5'-p TTG GGT AAC GCC AGG GTT TTC CCA GTC ACG OH-3', homologous to the *lac* Z' region in pUC and M13 plasmids) labeled with digoxigenin-11-ddUTP under standard assay reaction conditions, in redistilled water	1. Vial 6, Genius 5 Kit 2. Cat. No. 1585 754
Control Oligonucleotide, DIG-dUTP/dATP-tailed	2.5 pmol/µl oligonucleotide (30-mer, 5'-p TTG GGT AAC GCC AGG GTT TTC CCA GTC ACG OH-3', homologous to the *lac* Z' region in pUC and M13 plasmids) tailed with digoxigenin-11-dUTP/dATP under standard assay reaction conditions, in redistilled water	1. Vial 6, Genius 6 Kit
Labeled Control RNA	Digoxigenin-labeled "antisense" Neo RNA transcribed with T7 RNA polymerase, according to the standard protocol, from 4 µl (equivalent to 1 µg) *Pvu* II-linearized pSPT18 Neo-DNA	1. Vial 5, Genius 4 Kit 2. Cat. No. 1585 746

In addition to the DIG-labeled control, you will need the following reagents

	Description	Available from
DNA dilution buffer (for DNA and oligonucleotide probes only)	50 µg/ml herring sperm DNA, in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 (+25°C)	1. Vial 3, Genius 1 Kit 2. Vial 3, Genius 2 Kit 3. Vial 9, Genius 5 Kit 4. Vial 10, Genius 6 Kit 5. Vial 2, Genius 3 Kit
Blocking reagent	Blocking reagent for nucleic acid hybridization; white powder	1. Vial 11, Genius 1 Kit 2. Vial 6, Genius 3 Kit 3. Cat. No. 1096 176
NBT solution	75 mg/ml nitroblue tetrazolium salt in 70% (v/v) dimethylformamide	1. Vial 9, Genius 1 Kit 2. Vial 4, Genius 3 Kit 3. Cat. No. 1383 213 (dilute from 100 mg/ml)
X-phosphate solution	50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate), toluidinium salt in 100% dimethylformamide	1. Vial 10, Genius 1 Kit 2. Vial 5, Genius 3 Kit 3. Cat. No. 1383 221
Anti-DIG-alkaline phosphatase	Anti-digoxigenin [Fab] conjugated to alkaline phosphatase	1. Vial 8, Genius 1 Kit 2. Vial 3, Genius 3 Kit 3. Cat. No. 1093 274

Additionally required solutions

Except TE buffer, all of the following required solutions are available in a ready-to-use form in the **DIG Wash and Block Buffer Set (Cat. No. 1585 762)**. Bottle numbers for this set are indicated in parentheses. Alternatively, they can be prepared from separate reagents according to procedures described in Appendix B.

	Description
Washing buffer (Bottle 1; dilute 1:10 with H_2O)	100 mM maleic acid, 150 mM NaCl; pH 7.5 (+20°C); 3% (v/v) Tween 20
Maleic acid buffer (Bottle 2; dilute 1:10 with H_2O)	100 mM maleic acid, 150 mM NaCl; pH 7.5 (+20°C)
Blocking solution (Bottle 3; dilute 1:10 with 1X Maleic acid buffer)	1% (w/v) Blocking reagent for nucleic acid hybridization dissolved in Maleic acid buffer If necessary, treat with DEPC to destroy RNases. Autoclave the solution, and store at room temperature, +4°C, or –20°C. Blocking solution is cloudy and should not be filtered. It is stable for at least two weeks when stored at +4°C, but must then be brought to room temperature before use.
Detection buffer (Bottle 4; dilute 1:10 with H_2O)	100 mM Tris-HCl, 100 mM NaCl; pH 9.5 (+20°C) To increase the speed of colorimetric detection, 50 mM $MgCl_2$ may be added to the Detection buffer; however, filter the $MgCl_2$-containing buffer through a 0.45 µm membrane filter before use. This eliminates any precipitated $MgCl_2$, which can contribute to background if not removed.
TE buffer	10 mM Tris, 1 mM EDTA; pH 8.0 (+20°C)
DEPC-treated H_2O (for RNA probes only)	Sterile, distilled water treated with 0.1% diethylpyrocarbonate (DEPC)

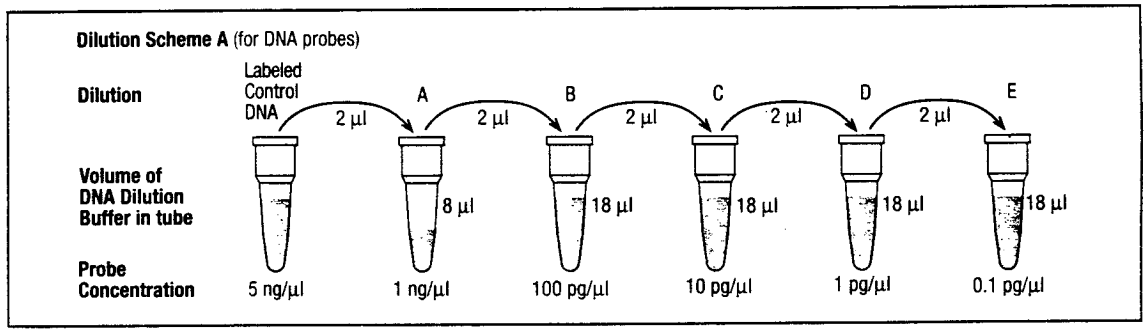
Procedure

❶ Make serial dilutions of the DIG-labeled control in DNA dilution buffer according to the appropriate dilution scheme. Or, for RNA controls, perform dilutions with DEPC-treated H_2O. (Use of siliconized tubes helps prevent adsorption of DNA to walls of the tubes.)

Dilution Scheme A (for DNA probes)

Labeled Control DNA Starting Concentration	Stepwise Dilution	Final Concentration (dilution name)	Total Dilution
5 ng/µl	2 µl/8 µl DNA dilution buffer	1 ng/µl (A)	1:5
1 ng/µl (dilution A)	2 µl/18 µl DNA dilution buffer	100 pg/µl (B)	1:50
100 pg/µl (dilution B)	2 µl/18 µl DNA dilution buffer	10 pg/µl (C)	1:500
10 pg/µl (dilution C)	2 µl/18 µl DNA dilution buffer	1 pg/µl (D)	1:5,000
1 pg/µl (dilution D)	2 µl/18 µl DNA dilution buffer	0.1 pg/µl (E)	1:50,000

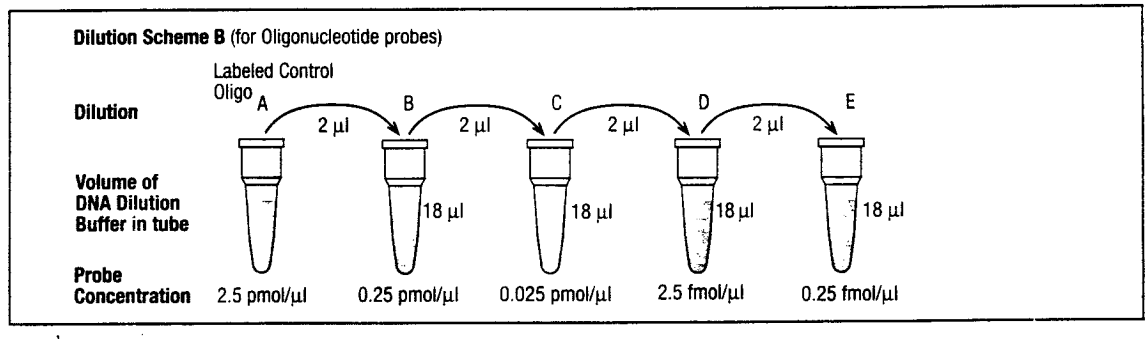
Dilutions A–E can be stored at –20°C for at least 1 year.



Dilution Scheme B (for Oligonucleotide probes)

DIG-tailed- or end-labeled Control Oligo Starting Concentration	Stepwise Dilution	Final Concentration (dilution name)	Total Dilution
2.5 pmol/µl (A)	2 µl/18 µl DNA dilution buffer	0.25 pmol/µl (B)	1:10
0.25 pmol/µl (dilution B)	2 µl/18 µl DNA dilution buffer	0.025 pmol/µl (C)	1:100
0.025 pmol/µl (dilution C)	2 µl/18 µl DNA dilution buffer	2.5 fmol/µl (D)	1:1,000
2.5 fmol/µl (dilution D)	2 µl/18 µl DNA dilution buffer	0.25 fmol/µl (E)	1:10,000

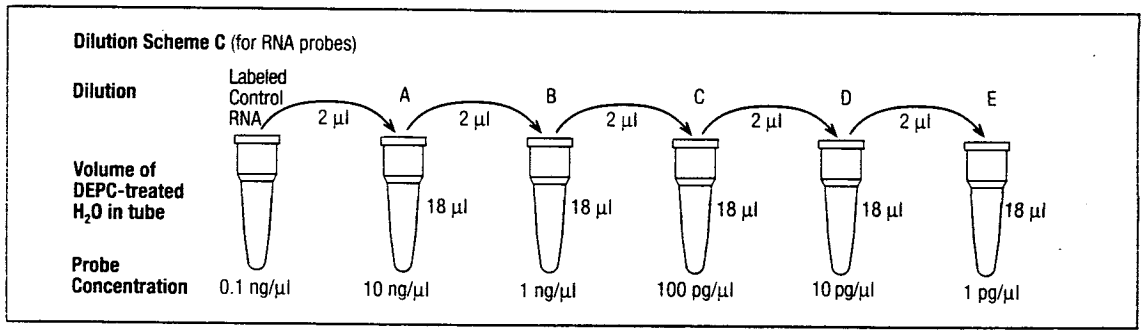
Dilutions A–E can be stored at –20°C for at least 1 year.



Dilution Scheme C (for RNA probes)

DIG-labeled Control RNA Starting Concentration	Stepwise Dilution	Final Concentration (dilution name)	Total Dilution
0.1 µg/µl	2 µl/18 µl H_2O	10 ng/µl (A)	1:10
10 ng/µl (dilution A)	2 µl/18 µl H_2O	1 ng/µl (B)	1:100
1 ng/µl (dilution B)	2 µl/18 µl H_2O	100 pg/µl (C)	1:1,000
100 pg/µl (dilution C)	2 µl/18 µl H_2O	10 pg/µl (D)	1:10,000
10 pg/µl (dilution D)	2 µl/18 µl H_2O	1 pg/µl (E)	1:100,000

Dilutions A–E can be stored at –70°C for at least 1 year.

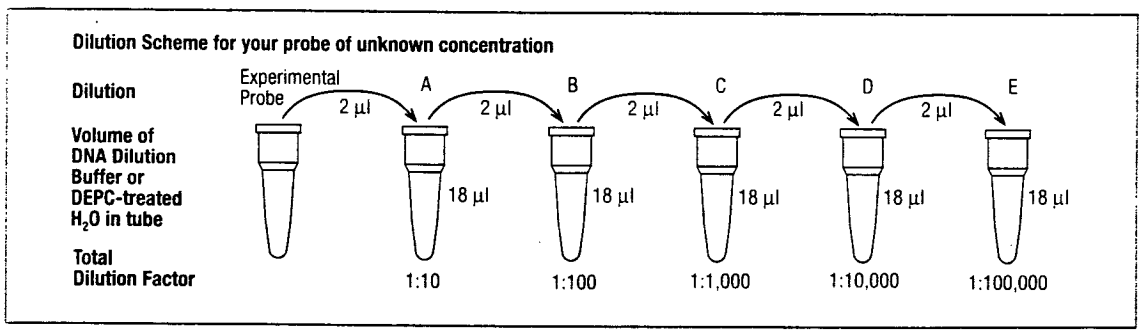


❷ Spot 1 µl of dilutions B–E onto a positively charged nylon membrane, marking the membrane lightly with a pencil to identify each dilution.

We recommend Boehringer Mannheim Nylon Membranes (Cat. Nos. 1209 272, 1209 299, 1417 240).

❸ Make serial ten-fold dilutions of the newly labeled experimental DNA probe (of unknown starting concentration) in DNA dilution buffer. Or, for RNA probes, perform dilutions with DEPC-treated H_2O.

If PCR was performed, additional dilutions of the experimental DNA may be necessary.



❹ Spot 1 µl of each of the dilutions made in step 3 onto the same nylon membrane, marking the membrane lightly with a pencil to identify each dilution. Mix dilutions well immediately before spotting on membrane.

❺ Fix the nucleic acids to the membrane by cross-linking with UV light or by baking for 30 minutes at +80°C.

❻ Wet the membrane with a small amount of Washing buffer.

❼ Incubate the membrane in Blocking solution for 5 minutes at room temperature.

❽ Mix 45 µl NBT solution and 35 µl X-phosphate solution in 10 ml of Detection buffer.
This freshly prepared Color Substrate Solution will be used in step 13. Protect from direct light before use.

❾ Dilute anti-DIG-alkaline phosphatase 1:5,000 in Blocking solution.

❿ Incubate the membrane in the diluted antibody for 10 minutes at room temperature.
The diluted antibody solution must cover the entire membrane.

⓫ Wash the membrane twice, 5 minutes per wash, in Washing buffer at room temperature.

⓬ Incubate the membrane in Detection buffer for 2 minutes.
Application of this buffer "activates" the alkaline phosphatase that is conjugated to the antibody.

⓭ Pour off the Detection buffer and add the Color Substrate Solution. Allow color development to occur in the dark for 30–60 minutes, monitoring occasionally. Do not shake the membranes.
Dilution D of the DIG-labeled control should be visible 30 minutes after adding the Color Substrate Solution. Since the incubations times are shortened in the interest of getting a quick assay result, the sensitivity of this assay may not be great enough to visualize the most diluted sample (Dilution E).

⓮ When the desired spots appear in sufficient intensity, stop the reaction by washing the membrane with 50 µl TE buffer or sterile H_2O for 5 min.

⓯ Compare spot intensities of the control and experimental dilutions to estimate the concentration of the experimental probe (See Figure 7).

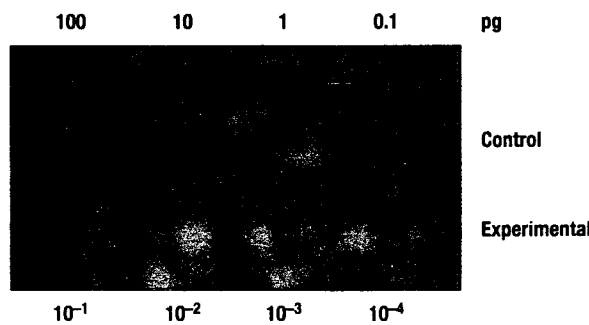


Figure 7. Estimating the Yield of DIG-labeled DNA. Dilutions of the Labeled Control DNA and the newly labeled (experimental) DNA were spotted on, fixed to, and directly detected on a Boehringer Mannheim Nylon Membrane with a chemiluminescent substrate. In this example, the intensity of the 10 pg Labeled Control DNA spot is approximately equal to that of the 10^{-3} dilution of the experimental DNA. From this, we calculate the amount of DIG-labeled DNA to be

$$10\ pg/\mu l \times 10^3 = 10{,}000\ pg/\mu l$$

The total yield of DNA is the concentration of the DIG-labeled DNA multiplied by the volume of probe resuspension. Because the DNA was resuspended in 50 µl TE buffer, the total yield is

$$10{,}000\ pg/\mu l \times 50\ \mu l = 500{,}000\ pg \text{ or } 500\ ng \text{ of labeled DNA probe.}$$

WHAT TO DO NEXT At this time, proceed to the "Hybridization" section of this User's Guide, which begins on page 41.

Chapter 6 *Purification of DIG-Labeled Nucleic Acids*

If desired, DIG-labeled nucleic acids can be purified by ethanol precipitation, Proteinase K treatment, or with a Quick Spin™ column.

Ethanol Precipitation

Products required

Name in procedure	Description	Availability
Glycogen solution	20 mg/ml glycogen in redistilled water	Cat. No. 901 393

Additionally required solutions

In addition to glycogen, you will need the following solutions.

Additional required solutions	Description
LiCl	4 M lithium chloride solution; 3 M sodium acetate or 3 M sodium chloride may be substituted for the LiCl; **do not use ammonium acetate.**
Ethanol	Absolute ethanol, chilled at –20°C; when 70% ethanol is indicated, dilute ethanol with redistilled water.
TE/SDS buffer	10 mM Tris-HCl, 1 mM EDTA; pH 7.0–8.0; containing 0.1% sodium dodecyl sulfate (SDS)

Procedure

1. Add 1 µl Glycogen solution to the reaction tube.
2. Precipitate the labeled nucleic acid with 0.1 volume of 4 M LiCl and 2.5–3.0 volumes of chilled Ethanol. Mix well and incubate at –70°C for 30 minutes.
3. Remove from the –70°C incubation and thaw briefly at room temperature. Centrifuge the reaction at 13,000 x g for 15 minutes in a microcentrifuge.
4. Decant the Ethanol and wash the pellet with 100 µl of 70% Ethanol. Centrifuge at 13,000 x g for 5 minutes in the microcentrifuge, then remove the 70% Ethanol.
5. Dry the pellet and resuspend in 50 µl of TE/SDS buffer. (Resuspend in H_2O if performing Proteinase·K digestion.) If not used immediately, store the labeled probe at –20°C (–70°C for RNA).

   The SDS may be left out if siliconized tubes are used during precipitation.

   Note: Resuspension of the digoxigenin-labeled pellet may require heating to +37°C for 10 minutes with frequent vortexing. Failure to adhere to these guidelines will result in the loss of approximately 20% of the digoxigenin-labeled DNA.

WHAT TO DO NEXT *For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.*

Proteinase K Treatment

In our laboratories, treatment of the DIG-labeled probe with Proteinase K after ethanol precipitation has increased signal-to-noise ratios on subsequent blots.

Products required

Name in procedure	Description	Available as
Proteinase K	20 mg/ml Proteinase K in 10 mM Tris-HCl, pH approx 7.5, containing calcium acetate	Cat. Nos. 1413 783, 1373 196, 1373 200

Additionally required solution

Additionally required solution	
H_2O	Sterile, distilled water

Procedure

1. After ethanol precipitation, resuspend the DIG-labeled probe in 50 µl H_2O.
2. Add $^1/_{10}$ volume Proteinase K, and incubate for 2 hours at +37°C.

   The resulting mixture can be used directly in any of the hybridization applications.

WHAT TO DO NEXT *For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.*

Purification of DNA Probes With a Quick Spin™ Column

DNA probes that have been labeled with DIG can be purified with Quick Spin columns.

Products required

Name in procedure	Description	Available as
Herring sperm DNA	10 mg/ml DNA from cod and herring sperm DNA; solution; sonicated and denatured (single-stranded fragments 120–3000 nucleotides in length) in 10 mM Tris-HCl 10 mM NaCl, 1 mM EDTA; pH 8.0	Cat. No. 1467 140
Quick Spin column	Quick Spin G-50 columns for biotinylated DNA purification (Prepacked, pre-spun Sephadex, G-50 [fine] columns, pre-swollen in SSC buffer [150 mM NaCl, 15 mM sodium citrate, 0.1% (w/v) SDS; pH 7.0])	Cat. Nos. 100 609, 100 611

Procedure

1. Add 5 µl (50 µg) Herring sperm DNA to the DIG-labeled DNA probe sample.
2. Allow the Quick Spin column unit to warm to room temperature.
3. Gently invert the Quick Spin column several times to resuspend the separation medium.
4. Remove the top cap from the Quick Spin column, then remove the bottom tip.

   The top cap must be removed first to avoid creating a vacuum and uneven flow of the column buffer. Allow the buffer to drain by gravity before proceeding.
5. Cut the bottom tip off one of the collection tubes, removing the bottom 5 mm. Place the Quick Spin column in this modified collection tube. Next, place this column/tube apparatus into an adaptor tube (*e.g.*, a sterile, disposable 16 x 125 mm round-bottom polystyrene collection tube with screw cap). Centrifuge at 1100 x *g* for 2 min. Discard the collection tube and the eluted buffer.

   Be sure to use a swinging-bucket rotor rather than a fixed-angle rotor. In a swinging-bucket rotor, the sleeves swing out as the speed of the centrifuge increases so that the force on the tube is always straight through the center instead of at an angle. In a fixed-angle rotor, the DNA sample is likely to slide down the sides of the tube instead of flowing through the separation medium. This results in poor retention of nucleotides.

   The modified collection tube allows excess buffer to flow into the adaptor tube. If the collection tube is not cut in this way, the small collection tube will over-flow with buffer and some buffer may be drawn back into the column, resulting in varying amounts of liquid obtained in the second spin.

6. Keeping the Quick Spin column in an upright position, very slowly and carefully apply the DNA sample (up to 50 µl) to the center of the column bed.

   Avoid applying the sample to the sides of the column; if this occurs, nucleotides flow around the separation medium and are not retained. Overloading the column (volume >50 µl) also results in nucleotides flowing around the separation medium and contaminating the DNA sample.

7. Keeping the Quick Spin column in an upright position, place the column in the second collection tube.

   Maintaining the column in an upright position is very important, especially after centrifugation of the sample. Tipping the column causes back-flow of the purified DNA sample, resulting in reduced DNA recovery.

8. Centrifuge at 1100 x *g* for 4 min.

9. Save the eluate from the second collection tube.

   This contains your purified DNA sample.

10. Discard the column.

WHAT TO DO NEXT *For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.*

Chapter 7 *General Considerations for Hybridization*

Please review this section of general hybridization considerations before proceeding with the Genius System. Several points are critical for successful use of the Genius System, especially when performing chemiluminescent detection. For general information on nucleic acid hybridization, see

> Sambrook, J., Fritsch, E.M. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Membrane Selection

The choice of membrane for use with the Genius System may be the most important parameter for success. Nylon, nitrocellulose, and PVDF membranes may be used. The choice of nylon or nitrocellulose membrane will depend on which detection method is chosen and whether stripping and reprobing will be performed. **Nitrocellulose membranes should not be used with any chemiluminescent substrate other than Lumi-Phos Plus. Also, nitrocellulose should not be used if stripping and reprobing will be performed because decolorizing reagents dissolve nitrocellulose.** Use the flow chart below to determine which type of membrane to use.

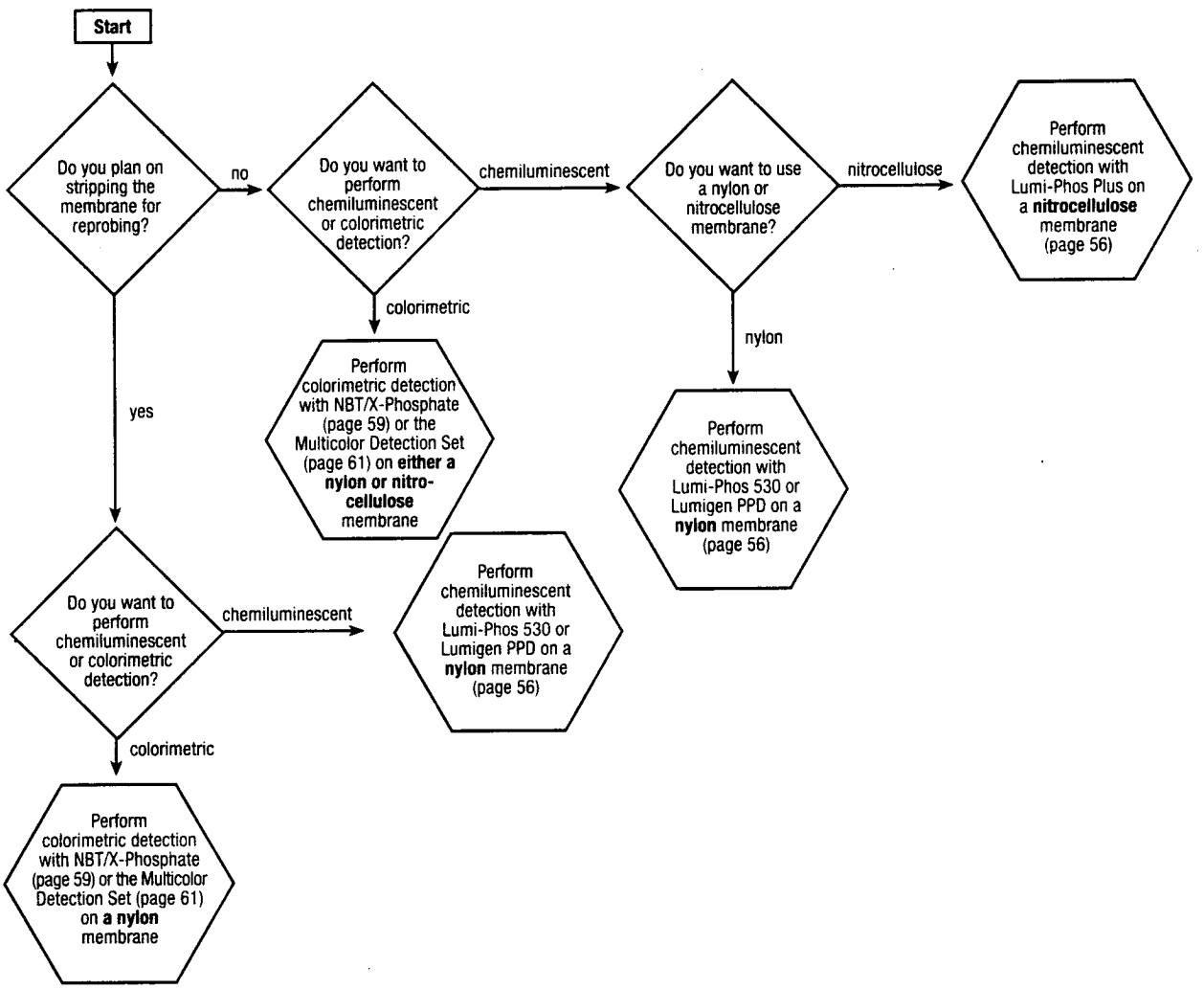


Figure 8. Flow chart for selecting the appropriate membrane.

Nylon membranes

For best results, use Boehringer Mannheim's Nylon membranes, positively charged (Cat. Nos. 1209 299, 1209 272, 1417 240) for the transfer. This membrane has an optimal charge density, allowing it to bind the nucleic acid tightly without producing high backgrounds. Our nylon membrane is also specifically tested with the Genius System to ensure optimal background characteristics. Other membranes that work well are Amersham's Hybond™-N and M.S.I.'s Magnagraph nylon membranes, but these are not tested with the Genius System and may produce high backgrounds due to lot-to-lot variability.

Nitrocellulose membranes

All nitrocellulose membranes tested work well with the Genius System, but will not work when performing chemiluminescent detection with Lumi-Phos 530 or Lumigen PPD or when stripping and reprobing membranes. Chemiluminescent detection can be performed on nitrocellulose membranes, but **only** with Lumi-Phos Plus.

Probe Concentration

When working with a radioactive probe, it is common to refer to the "specific activity" (the number of cpms or dpms) of a probe in a hybridization reaction. For digoxigenin-labeled probes, however, the concept of probe concentration (*e.g.*, ng/ml) in a hybridization reaction is much more meaningful than specific activity. The approximate probe concentration can be determined from the sections on estimating the yield of the DIG-labeled DNA, oligonucleotide, or RNA, which appear in several of the labeling sections.

NOTE: If chemiluminescent detection is to be performed, **the concentration of a DIG-labeled DNA probe must not exceed 25 ng/ml**; use of a probe concentration >25 ng/ml will result in high background. Higher concentrations may be used for northern blots.

Optimization of the probe concentration – the "mock" hybridization

To prevent background problems, we recommend that the probe concentration be optimized before hybridization. The mock hybridization is especially important when performing chemiluminescent detection.

The mock hybridization is carried out by incubating small membrane pieces (without DNA) with different probe concentrations in the hybridization solution and afterwards detecting according to standard procedure (page 56 or 59).

For example:

Probe type	Concentration in the hybridization solution		
DNA/RNA probes	1 µl*/ml	3 µl*/ml	5 µl*/ml
End-labeled oligonucleotide	1 pmol/ml	3 pmol/ml	10 pmol/ml
Tailed oligonucleotide	0.1 pmol/ml	0.5 pmol/ml	2 pmol/ml

*from the labeling reaction

The highest probe concentration that gives an acceptable background should be used for the hybridization experiment.

Hybridization and Washing Conditions

We have found that DIG-labeled probes demonstrate the same hybridization kinetics as radiolabeled probes. Hybridization and washing conditions for DIG-labeled probes do not differ substantially from those of radiolabeled probes. As in the case of radiolabeled nucleic acid probes, the optimal hybridization and wash conditions for each DIG-labeled probe must be determined experimentally. In this User's Guide, we provide recommendations for hybridization and washing conditions. Use the conditions given with each application as a starting point. It may then be necessary to optimize conditions to obtain maximum sensitivity with your probe.

Labeled probes can hybridize non-specifically to sequences that bear homology but are not entirely homologous to the probe sequence. Such hybrids are less stable than perfectly matched hybrids. They can be dissociated by performing washes of various stringencies. The stringency of the washes can be manipulated by varying the salt concentration and temperature. For some applications, the stringency of the washes should be higher. However, we recommend that you hybridize stringently (*i.e.*, optimize hybridization conditions) rather than wash stringently.

Prehybridization/Hybridization Solutions

The presence of the DIG label does not alter the hybridization characteristics of a nucleic acid probe. Therefore, the prehybridization/hybridization solutions and conditions (temperature, stringency) commonly used in molecular biology techniques are also compatible with the Genius System. The main consideration when choosing a buffer system is the subsequent immunological detection and potential backgrounds that might develop.

In our experience, optimal results have been obtained with the following buffers:

Standard buffer	Standard buffer + 50% formamide	High SDS buffer (Church's buffer)	DIG Easy Hyb
5X SSC 0.1% (w/v) N-lauroylsarcosine 0.02% (w/v) SDS 1% Blocking Reagent	50% Formamide, deionized 5X SSC 0.1% (w/v) N-lauroylsarcosine 0.02% (w/v) SDS 2% Blocking Reagent	7% SDS 50% Formamide, deionized 5X SSC 2% Blocking Reagent 50 mM Sodium Phosphate, pH 7.0 0.1% (w/v) N-lauroylsarcosine	Cat. No. 1603 558 (a nontoxic solution used like a formamide-based hybridization buffer)

Figure 9. You may use sealable containers, glass baking dishes (covered with plastic wrap or foil), or heat-sealable plastic bags for hybridization and washing.

Storage and Reuse of Hybridization Solutions

One of the advantages of the Genius System is the stability of the labeled probe. After hybridization against the blotted target, the hybridization solution still contains large amounts of unannealed DIG-labeled probe. Simply pour the solution into a plastic tube, seal the cap, and store at –20°C for DNA probes or –70°C for RNA probes. DIG-labeled probes are stable for at least 1 year when stored in this manner. For reuse, thaw and denature by heating to +95°C for 10 minutes. If the hybridization solution contains 50% formamide (the flash point of pure formamide is +68°C), denature at +68°C for 10 minutes.

Stripping and Reprobing

With the Genius System, membranes can be easily stripped and reprobed, especially when probes have been labeled with alkali-labile DIG-dUTP. To do this, refer to the procedures on page 65.

WHAT TO DO NEXT *At this time, proceed to the appropriate application in the "Hybridization" section of the User's Guide.*

Southern Blotting

With Lumi-Phos 530, Lumi-Phos Plus, or Lumigen PPD, the Genius System can detect 0.1 pg of homologous DNA in a Southern blot format on a nylon membrane. This corresponds to the detection of a single-copy gene in <1 µg of human genomic DNA. The procedures described here are used routinely in our labs and have been found to give optimal results in Southern blotting, particularly in genomic Southern blotting.

Required solutions

Solutions required for Southern blotting are listed below. **Refer to Appendix B for details on preparing these required solutions.**

Required solution	Description
HCl	250 mM HCl
H_2O	Sterile, distilled water
Denaturing solution	0.5 N NaOH, 1.5 M NaCl
Neutralization solution	1.0 M Tris-HCl, pH 8.0; 1.5 M NaCl
20X SSC buffer	3 M NaCl, 300 mM sodium citrate; pH 7.0
5X SSC buffer	750 mM NaCl, 75 mM sodium citrate, pH 7.0
Prehybridization solution *added from the blocking reagent stock solution (100 mM maleic acid; 150 mM NaCl, pH 7.5, containing 10% [w/v] Blocking reagent for nucleic acid hybridization)	**Prepare one of the following** (see Table 7 on page 46 for hybridization solution requirements, and see Appendix B for buffer preparation): *Standard buffer* 5X SSC, 1.0% (w/v) Blocking reagent for nucleic acid hybridization*, 0.1% N-lauroylsarcosine, 0.2% sodium dodecyl sulfate (SDS) *Standard buffer + 50% formamide* 5X SSC, 2.0% (w/v) Blocking reagent for nucleic acid hybridization*, 0.1% N-lauroylsarcosine, 0.2% sodium dodecyl sulfate (SDS), 50% formamide (deionized) *High SDS buffer* 5X SSC, 2.0% (w/v) Blocking reagent for nucleic acid hybridization*, 50 mM sodium phosphate (pH 7.0), 0.1% N-lauroylsarcosine, 7% sodium dodecyl sulfate (SDS), 50% formamide (deionized) *DIG Easy Hyb* Ready-to-use hybridization solution (Cat. No. 1603 558)
Hybridization solution	DIG-labeled probe diluted in prehybridization solution
2X wash solution	2X SSC containing 0.1% SDS
0.5X wash solution	0.5X SSC containing 0.1% SDS

Gel Electrophoresis

Restriction-digest the DNA. Prepare an agarose gel of appropriate composition, using a high-purity or nucleic acid-grade agarose, such as our low EEO agarose (Cat. Nos. 100 437, 100 439). Use either Tris-Borate-EDTA buffer (TBE; Cat. No. 100 957) or Tris-Acetate-EDTA buffer (TAE; Cat. No. 100 953). Run the digest on the gel. If desired, the gel may be stained with ethidium bromide to visualize DNA fragments and to confirm subsequent Southern transfer to the membrane.

Southern Transfer

The transfer of DNA from the gel to the membrane can be accomplished by one of a number of common procedures; however, the following procedures are routinely used in our lab and provide optimal detection sensitivity.

Depurination (optional)

Controlled acid treatment depurinates DNA, improving the transfer of large DNA fragments (>10 kb) from gel to membrane in the Southern transfer procedure. Depurination is an optional treatment. If you are transferring small DNAs (<10 kb) or detecting small DNA fragments in a genomic digest, it may not be necessary to depurinate the DNA. Avoid excessive acid treatment, which could result in poor detection sensitivity.

1. Submerge the agarose gel in 250 mM HCl for 10 minutes (with shaking) at room temperature. Do not exceed 10 minutes.
2. Rinse the gel with H_2O before proceeding to the Southern transfer.

Denaturation and neutralization

1. Submerge the agarose gel in Denaturing solution for 30–60 minutes at room temperature. Shake gently. This incubation denatures the DNA target prior to transfer.
2. Submerge the gel in Neutralization solution for 30–60 minutes at room temperature to neutralize the gel.
3. Prepare membrane filters for Southern transfer according to manufacturer's recommendations. Boehringer Mannheim Nylon membranes, positively charged (Cat. Nos. 1209 299, 1209 272, 1417 240) require no additional preparation steps.
4. Blot the DNA from the gel by capillary transfer to the membrane, using 10X or 20X SSC buffer (Cat. No. 100 949). Blot overnight to ensure efficient transfer of the DNA. Alternatively, the DNA can be vacuum-blotted onto the membrane; vacuum-blotting can be accomplished in 1–2 hours.

DNA Fixation

DNA can be efficiently bound to the nylon or nitrocellulose membrane by the following procedure.

Procedure

1. Rinse the membrane in 5X SSC buffer for 1 minute at room temperature. This wash removes gel debris and particulate contaminants that were introduced as a result of the transfer process.
2. Carefully place the damp membrane on a piece of blotting paper (*e.g.*, Whatman 3MM). Bake in an oven at +80°C for 1 hour or at 120°C for 15–30 min. When using a nitrocellulose membrane, bake in a vacuum oven to prevent the spontaneous combustion of the nitrocellulose.
3. After fixation, the membrane can be used in prehybridization, or stored dry at +4°C in a desiccation chamber for future detection.

U.V. crosslinking may also be used to fix DNA to the membrane. Follow the manufacturer's specifications when doing this. If UV-crosslinking was performed, rinse the membrane after the fixation process.

Prehybridization and Hybridization

Prehybridization prepares the membrane for probe hybridization by blocking non-specific nucleic acid-binding sites on the membrane. This ultimately serves to lower background. Many different prehybridization solutions have been described in the literature. However, the prehybridization solutions described here combine efficient blocking with ease of use.

As with any probe, optimal hybridization conditions for DIG-labeled probes must be determined experimentally. We strongly recommend that the time be taken to optimize each DIG-labeled probe (see the mock hybridization on page 42). The time taken for optimization will result in cleaner results and, ultimately, time savings, especially if a probe will be reused many times.

Procedure

1. Place the blot in a hybridization bag containing 20 ml prehybridization solution per 100 cm^2 of membrane surface area. Seal the bag, and prehybridize at the anticipated hybridization temperature for 2 hours.
   Longer prehybridization times are acceptable. Several membranes can be processed in the same sealed bag as long as there is sufficient prehybridization solution to cover all the membranes, and the membranes can move freely in the bag.
   The optimal hybridization temperature for a specific probe will depend on the length of the probe and on the extent of sequence homology with the target sequence; therefore, it must be determined empirically. See Table 7 for recommended temperatures for different types of probes and different hybridization solutions.

Probe type	Probe concentration	Hybridization solution	Temperature for prehybridization and hybridization
DNA	5–25 ng/ml	Standard buffer	Hybridize overnight at +50°C–68°C
		Standard buffer + 50% formamide	Hybridize overnight at +42°C–55°C
		DIG Easy Hyb	Hybridize overnight at +42°C–55°C
		High SDS buffer	Hybridize overnight at +42°C–55°C
		High SDS buffer without 50% formamide*	Hybridize overnight at +55°C
		*(recommendation for Single Locus Fingerprinting probes)	
RNA	100 ng/ml	Standard buffer + 50% formamide	Hybridize overnight at +37°C–55°C
		DIG Easy Hyb	Hybridize overnight at +37°C–55°C
Oligonucleotides		Standard buffer	Hybridize for 1–6 h; hybridization temperature varies considerably and can be approximated by considering probe length and G plus C content. Hybridization with a tailed oligonucleotide should be performed with 0.1 mg/ml Poly(A) (in the prehybridization and hybridization solution) to prevent nonspecific hybridization signals. Additionally, 5 µg/ml of poly(dA) may be added for further blocking.
tailed	0.1–2 pmol/ml		
end labeled	1–10 pmol/ml		

*The conditions given here are stringent conditions applicable if probe and target have 100% homology and a GC content of about 50%.

Table 7. Optimal hybridization conditions for different probe types.

❷ When using double-stranded DNA probes, heat in a boiling water bath for 10 minutes to denature the DNA. Chill directly on ice.

Single-stranded RNA probes and oligonucleotide probes do not require denaturation prior to dilution unless extensive secondary structure is predicted from the sequence. Prepare the hybridization solution in a volume sufficient to allow each blot to move freely in the solution (*e.g.*, 10 ml for a 10 x 10 cm, or 25 ml for a ≥20 x 20 cm membrane).

❸ Dilute the probe in hybridization solution. See Table 7 for optimal probe concentrations.

❹ Discard the prehybridization solution from the bag. Add the hybridization solution containing the DIG-labeled probe. Allow the probe to hybridize. See Table 7 for selecting a hybridization solution and temperature.

The required amount of hybridization solution for a 100 cm^2 blot is at least 20 ml.

❺ At the end of the hybridization, pour the hybridization solution from the bag into a tube (with cap) that can withstand freezing and boiling (*e.g.*, 50 ml polypropylene).

This hybridization solution contains unannealed DIG-labeled probe. The entire solution can be reused in future hybridization experiments. Label and date the tube, and store DNA probe solutions at –20°C and RNA probe solutions at –70°C. DIG-labeled probes stored in this manner are stable for at least 1 year. For reuse, thaw and denature by heating to +95°C for 10 minutes. If the hybridization solution contains 50% formamide (the flash point of pure formamide is +68°C), denature at +68°C for 10 minutes.

❻ Wash the membrane twice, 5 minutes per wash, in 2X wash solution at room temperature.

These washes (steps 6 and 7) remove unbound probe, which will lead to high backgrounds if not removed.

❼ Wash the membrane twice, 15 minutes per wash, in 0.5X wash solution.

Long probes (>100 bp) should be washed at +65°C. For shorter probes, the wash temperature must be determined empirically.

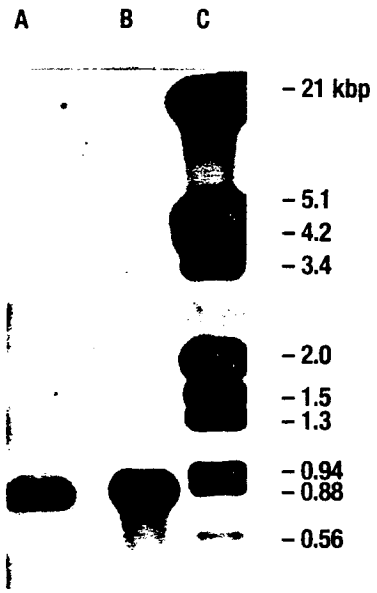


Figure 10. A typical Southern blot. Southern blot analysis of 10 µg *Hin*d III-digested plant genomic DNA of transgenic tobacco ST1a containing a single copy of the *npt*-II gene (gift from M. Saul, personal communication), which was obtained by PEG-mediated direct gene transfer (M. Saul, *et al.*, 1988). The DNA was transferred to the positively charged Nylon Membrane from Boehringer Mannheim and hybridized with a DIG-11-dUTP- labeled *Hin*d III fragment of the plasmid pSHI 913 (M. Schnorg, *et al.*, 1991) at a concentration of 25 ng DIG-labeled DNA/ml hybridization solution. The hybridization was performed in a hybridization oven in the presence of 50% formamide as described by Neuhaus-Url and Neuhaus (Transgenic Research, in press).
A. 10 µg of *Hin*d III-restricted plant DNA of ST1a releasing 1 copy of the 800 bp *npt*-II coding region.
B. 10 pg of the *Hin*d III fragment of pSHI 913 reflecting 1 gene copy.
C. 40 ng of DIG-labeled Molecular Weight Marker III (Boehringer Mannheim).
The exposure time was 0.5 h. The Lumigen® PPD incubation time (time elapsed between the Lumigen PPD preincubation and the start of the X-ray exposure) was 24 h.

Data were kindly provided by Dr. G. Neuhaus-Url, ETH, Zurich, Switzerland.

WHAT TO DO NEXT *At this time, proceed to the "Detection" division of this User's Guide, which begins on page 56.*

DNA Dot Blotting

Dot blots and slot blots are rapid methods for the qualitative screening of DNA. Target DNA samples may be purified DNA, cell lysate, or PCR-amplified DNA.

The same hybridization and detection procedures used with Southern blots are also performed on DNA dot blots; therefore, proceed to the Southern prehybridization and hybridization procedures (page 45) after preparing the dot blot.

Required solutions

Products and solutions that are required for the hybridization of DNA, but not specifically required for the dot blotting procedure given here, are listed in the Southern blotting application (page 44).

Reagent/Solution	Description	Source
DNA dilution buffer	50 µg/ml herring sperm DNA; 10 mM Tris/HCl, pH 8.0; 1 mM EDTA, pH 8.0	

Procedure

1. Prepare a dilution series of your DNA target in suitable amounts.
2. Denature the DNA target in the dilutions for 10 min at +95°C, and chill immediately on ice.
3. Mark the membrane lightly with a pencil to identify each dilution before spotting.
   We recommend Boehringer Mannheim Nylon Membranes, positively charged.
4. Dispense 1 µl of each dilution onto the membrane. Mix dilutions well before dotting on membrane.
5. Fix the DNA to the membrane by UV crosslinking or baking in an oven at 80°C for 1 h or at +120°C for 30 min.

Figure 11. Chemiluminescent Dot Blot showing HLA class II typing. Human genomic DNAs from 48 patients were PCR amplified and blotted onto a nylon membrane. A HLA DRBI 01 sequence-specific oligonucleotide was 3'-end labeled with DIG-11-ddUTP by Terminal transferase and hybridized to the membrane. After washing, chemiluminescent detection was achieved with Anti-DIG-alkaline phosphatase antibody and Lumigen® PPD chemiluminescent substrate. The blot was exposed to X-ray film for 15 min.

Data were kindly provided by Dr. A. Moine-Grenoble Transfusion, La Tronche, France.

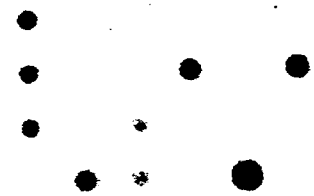
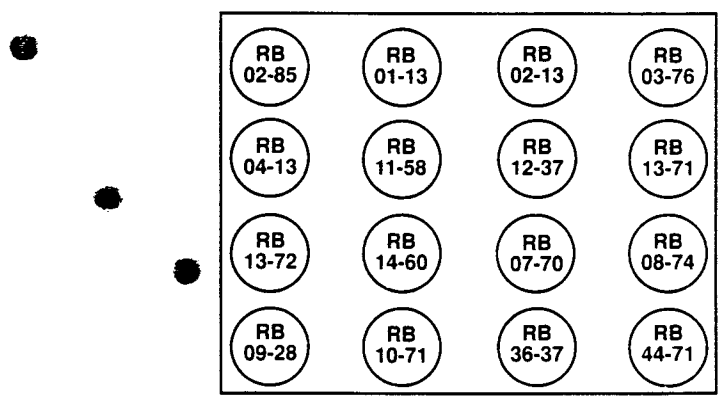


Figure 12: HLA-DR genotyping by chemiluminescent reverse Dot Blot. Sixteen sequence-specific oligonucleotides (SSOs) were blotted onto a nylon membrane. PCR-amplified target DNA (HLA-DR gene, second exons) corresponding to one individual was 3'-labeled with DIG-11-ddUTP by Terminal transferase, and hybridized to the immobilized SSOs. After washing, chemiluminescent detection of specific hybridization was achieved with anti-DIG-alkaline phosphatase antibody and Lumigen® PPD. The HLA-DR genotyping of this individual was found to be HLADRB1*01–DRB1*07.

Data were kindly provided by Dr. J.F. Eliaou – Laboratoire d'Immunologie, Montpellier, France.



WHAT TO DO NEXT *Hybridize the samples on the dot blot according to the "Prehybridization and Hybridization" procedure described in the "Southern Blotting" application, which begins on page 44.*

Colony and Plaque Hybridizations

The Genius System provides a sensitive and rapid method for detecting positive colonies or plaques in a heterogeneous background. Colony and plaque hybridizations have been developed to allow rapid screening of bacterial and phage recombinant genomic libraries for specific DNA sequences. The bacterial colonies or phage particles are transferred to a nylon membrane. Alkaline treatment serves to lyse the colonies or disassemble the phage particles. The denatured DNA is then immobilized on the membrane. A digoxigenin-labeled DNA, RNA, or oligonucleotide probe is used for hybridization. Detection is carried out with a colorimetric or chemiluminescent immunoassay.

Required solutions

Refer to Appendix B for details on preparing these additionally required solutions.

Required solutions	Description
Denaturation solution 1 (for plaque hybridization)	0.5 N NaOH, 1.5 M NaCl
Denaturation solution 2 (for colony hybridization)	0.5 N NaOH, 1.5 M NaCl, 0.1% SDS
Neutralization solution 2 (for colony and plaque hybridization)	1.0 M Tris-HCl, pH 7.5; 1.5 M NaCl
Prehybridization solution *added from the blocking reagent stock solution (100 mM maleic acid; 150 mM NaCl, pH 7.5, containing 10% [w/v] Blocking reagent for nucleic acid hybridization)	**Prepare one of the following** (see Table 7 for hybridization solution requirements, and see Appendix B for buffer preparation): *Standard buffer* 5X SSC, 1.0% (w/v) Blocking reagent for nucleic acid hybridization*, 0.1% N-lauroylsarcosine, 0.2% sodium dodecyl sulfate (SDS) *Standard buffer + 50% formamide* 5X SSC, 2.0% (w/v) Blocking reagent for nucleic acid hybridization*, 0.1% N-lauroylsarcosine, 0.2% sodium dodecyl sulfate (SDS), 50% formamide (deionized) DIG Easy Hyb Ready-to-use hybridization solution (Cat. No. 1603 558)
Hybridization solution	DIG-labeled probe diluted in prehybridization solution
2X SSC buffer	300 mM NaCl, 30 mM sodium citrate; pH 7.0
3X wash solution	3X SSC containing 0.1% SDS
2X wash solution	2X SSC containing 0.1% SDS
0.5X wash solution	0.5X SSC containing 0.1% SDS

Any type of DIG-labeled DNA, RNA, or oligonucleotide probe can be used for colony and plaque hybridizations. To avoid nonspecific hybridization, use a probe that does not contain any sequences homologous to the vector. The optimal hybridization temperature and probe concentration must be determined empirically. Table 8 offers general guidelines.

Probe type	Probe concentration	Hybridization solution	Temperature for prehybridization and hybridization
DNA	5–25 ng/ml	Standard buffer	Hybridize overnight at +68°C.
		Standard buffer + 50% formamide	Hybridize overnight at +42°C.
		DIG Easy Hyb	Hybridize overnight at +42°C.
RNA	100 ng/ml	Standard buffer + 50% formamide	Hybridize overnight at +50°C.
		DIG Easy Hyb	Hybridize overnight at +50°C.
Oligonucleotide tailed end labeled	 0.1–2 pmol/ml 1–10 pmol/ml	Standard buffer	Hybridize for 1–6 h; hybridization temperature varies considerably and can be approximated by considering probe length and G plus C content. (To determine the T_m, add 4°C for each G or C and 2°C for each T or A). To prevent non-specific hybridization signals, hybridization with a tailed oligonucleotide should be performed with 0.1 mg/ml Poly(A) in the prehybridization and hybridization solution. Additionally, 5 µg/ml Poly d(A) may be added for further blocking.

*The conditions given here are **stringent** conditions applicable if probe and target have 100% homology and a GC content of about 50%.

Table 8: Optimal hybridization conditions for different probe types.

Preparation of the Plaque or Colony Lift

1. Plate phages and lawn cells (or bacteria for colony screening) at near confluence on a 10 cm LB agar plate. Allow the plaques to grow overnight at +37°C. Allow bacterial colonies to grow until they have reached a diameter of 1–2 mm.
2. Chill the plates for 1 h at +4°C.
3. Place a nylon membrane on the cold agar plate for 1 min for a primary filter, or for 3 min for a duplicate filter. Label the filter asymmetrically with a needle to record the orientation of the filter on the plate. Store the original plates at +4°C. Replicas of plaque libraries are made directly this way.

   Note: Do not perform nonradioactive detection of colonies grown on nylon membranes; this can lead to false-positive signals. After the colony lift, the colonies can be recovered by placing the master plate back in the +37°C incubator for a few hours.

Processing on the Filter

1. Place the plaque lifts on dry blotting paper for 5–10 min.

   The plaque particles bind to the filter as it dries.
2. Place 3 sheets of blotting paper (approximately the size of the membrane) side by side on plastic wrap, and saturate them with Denaturation solution 1 or 2, Neutralization solution 2, or 2X SSC.
3. Place the plaque lifts (plaque side up) for 5 min on a blotting paper saturated with Denaturation solution 1. Place colonies (colony side up) on blotting paper saturated with Denaturation solution 2 for 15 min. Do not allow any solution to remain on top of the membrane.
4. Place the plaque or colony lifts on the blotting paper with Neutralization solution 2 for 5 min, and transfer to the 2X SSC-saturated blotting paper for 15 min.
5. Fix the DNA on the wet membranes by UV-crosslinking or baking onto the nylon membranes for 15–30 min at +120°C.
6. Only for colony hybridization is the removal of cellular debris necessary. To remove background on colony hybridizations, incubate the filter in 3X wash solution with shaking for 1–3 h at +68°C. Gently wipe the surfaces with a moistened towel.

   This removes cellular debris, which could lead to high background if not removed.

   If you expect background to be unusually high, treat the filters with Proteinase K (20 µg/ml) for 1 h at +37°C. For later immunological detection, it will be necessary to inactivate the Proteinase K by incubating the filter for 5 min in PMSF (40 µg/ml) at room temperature, performing two short washes in 2X SSC. **Note:** PMSF is extremely harmful if swallowed, inhaled, or adsorbed through the skin. Pefabloc® SC (Cat. No. 1429 868, 1429 876) is a safer alternative.

   The membrane can be used directly for hybridization or stored for later use. See Table 8 for prehybridization temperatures.

Hybridization

1. Place the filter in a hybridization bag or box containing at least 20 ml prehybridization solution for a 10 cm membrane disk. Seal the bag, and prehybridize at the hybridization temperature for at least 2 h with gentle shaking.

   Several filters can be processed in the same container as long as they are all covered and able to move freely in the solution.
2. Dilute the probe in hybridization solution. See Table 8 for recommended probe concentration.

   If a double-stranded DNA probe is being used, heat-denature it in a boiling water bath for 10 min before dilution.

   Single-stranded RNA probes and oligonucleotide probes do not require denaturation prior to dilution unless extensive secondary structure is predicted from the sequence.
3. Discard the prehybridization solution, and add the hybridization solution containing the DIG-labeled probe. Allow the probe to hybridize while gently shaking to redistribute the solution. See Table 8 for hybridization temperatures.

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❹ At the end of the hybridization, pour the hybridization solution from the bag into a tube (with cap) that can withstand freezing and boiling (*e.g.*, a 50 ml polypropylene tube).

The entire solution can be reused several times in future hybridization experiments. Label and date the tube, and store DNA probe solutions at –20°C, or RNA probe solutions at –70°C. DIG-labeled probes stored in this manner are stable for at least 1 year. For reuse, thaw and denature by heating to +95°C for 10 min. If the hybridization solution contains formamide (the flashpoint of pure formamide is +68°C), denature at +68°C for 10 min.

Stringency Washes

❶ After hybridization, wash the filters twice, 5 min per wash, in 2X wash solution at room temperature.

❷ Wash the filters twice, 15 min per wash, in 0.5X wash solution. Wash long probes (>100 bp) at +68°C. For shorter probes, the washing temperature should be the same as the hybridization temperature.

The filters can be used immediately for detection, or the filters can be air-dried and stored for later detection.

Note on subsequent detection

The filters can be detected with a colorimetric, chemiluminescent, or multicolor detection assay. The spotty background sometimes observed with chemiluminescent detection can be suppressed by using a buffer that does not contain Mg^{2+} ions (Detection buffer). In addition, we recommend that you briefly centrifuge the antibody vial and remove aliquots from the top. When performing plaque hybridizations, we recommend a second plaque lifting so that you can compare the two membranes and easily distinguish between positive and false signals.

WHAT TO DO NEXT *Proceed to the "Detection" division of this User's Guide, which begins on page 56.*

References

1. Grunstein, M. and Hogness, D.S. (1975) *Proc. Natl. Acad. Sci. USA* **72**:3961.
2. Benton, W.D. and Davis, R.W. (1977) *Science* **196**:180.

Northern Blotting

When performing northern blots with the Genius System, the same parameters must be considered to determine hybridization conditions. Like northern blotting with ^{32}P-labeled probes, northern blotting with DIG tends to be more difficult than Southern blotting or colony/plaque hybridizations. Nonetheless, the Genius System can be readily used to detect RNA on a membrane.

Probe Preparation

As is the case with radioactive probes, DIG-labeled RNA probes demonstrate stronger signals and less non-specific hybridization than DNA probes on northern and Southern Blots.

If a DNA probe must be used, we recommend that you use the High SDS hybridization buffer or DIG Easy Hyb to reduce background. See Table 9 for details on hybridization solutions for different probe types.

Optimization of the Probe Concentration

Optimize the probe concentration before all hybridization experiments. This is necessary to avoid background staining, and it can be easily performed with a series of mock hybridizations, where increasing concentrations of DIG-labeled probes are incubated with naked pieces of membrane or hybridized to dots of homologous DNA or RNA. This procedure is described on page 42.

Avoidance of RNase Contamination

Throughout the northern blot experiment, be careful to avoid the introduction of RNases, as RNA is susceptible to degradation even after its immobilization on a nylon membrane. We recommend sterilization of all solutions and containers that will come in contact with the RNA or northern blot. In addition to autoclaving, treat solutions and containers with DEPC (diethylpyrocarbonate).

Throughout the experiment, use forceps whenever possible, and wear gloves.

Optimal Blotting Conditions

Salt concentrations between 10X and 20X SSC give equivalent results for the transfer of RNA from a 1% agarose formaldehyde gel to a nylon membrane. The optimal blotting duration is overnight at room temperature.

Required solutions

Required solution	Description
Prehybridization solution	**Prepare one of the following** (see Table 9 for hybridization solution requirements, and see Appendix B for buffer preparation):
	High SDS buffer 5X SSC, 2.0% (w/v) Blocking reagent for nucleic acid hybridization*, 50 mM sodium phosphate (pH 7.0), 0.1% N-lauroylsarcosine, 7% sodium dodecyl sulfate (SDS), 50% formamide (deionized)
*added from the blocking reagent stock solution (100 mM maleic acid; 150 mM NaCl, pH 7.5, containing 10% [w/v] Blocking reagent for nucleic acid hybridization)	*DIG Easy Hyb* Ready-to-use hybridization solution (Cat. No. 1603 558)
	Standard buffer + 50% formamide 5X SSC, 2.0% (w/v) Blocking reagent for nucleic acid hybridization*, 0.1% N-lauroylsarcosine, 0.2% sodium dodecyl sulfate (SDS), 50% formamide (deionized)
	Standard buffer 5X SSC, 1.0% (w/v) Blocking reagent for nucleic acid hybridization*, 0.1% N-lauroylsarcosine, 0.2% sodium dodecyl sulfate (SDS)
Hybridization solution	DIG-labeled probe diluted in prehybridization solution
2X wash solution	2X SSC containing 0.1% SDS.
0.5X wash solution	0.5X SSC containing 0.1% SDS.
20X SSC	3 M NaCl, 300 mM sodium citrate; pH 7.0; treated with DEPC
10X SSC	1.5 M NaCl, 150 mM sodium citrate; pH 7.0; treated with DEPC

Controls

A DIG-labeled anti-sense Actin RNA hybridization probe (Cat. No. 1498 045) is available for evaluating the quality and quantity of your RNA.

Northern Transfer

1. After electrophoresis in a standard formaldehyde gel, equilibrate the gel in 20X SSC (DEPC-treated) for 2 x 15 min.
2. Prepare a membrane filter. Wear powder-free gloves when handling the membrane, and manipulate the membrane by applying forceps to the edges.

   For best results, use Boehringer Mannheim's Nylon Membranes (Cat. Nos. 1209 299, 1209 272, 1417 240) for the transfer. This membrane has an optimal charge density, allowing it to bind the RNA tightly without producing high backgrounds. Our nylon membrane is also specifically tested with the Genius System to ensure optimal background characteristics. Other membranes that work well are Amersham's Hybond-N and M.S.I.'s Magnagraph nylon membranes, but these are not tested with the Genius System and may produce backgrounds due to lot-to-lot variability.
3. Blot the RNA from the gel by capillary transfer overnight at +4°C or 4 h at room temperature with 10X or 20X SSC (DEPC-treated).
4. UV-crosslink or bake the membrane at 80°C for 1 h or at +120°C for 30 min.

Prehybridization and Hybridization

Before hybridization, determine the optimal probe concentration according to the mock hybridization protocol on page 42. Table 9 gives general guidelines for probe concentrations and hybridization temperatures.

1. Place the blot in a hybridization bag containing 20 ml prehybridization solution per 100 cm^2 of membrane surface area. Seal the bag, and prehybridize at the anticipated hybridization temperature for at least 1 h.

   Longer prehybridization times are acceptable.

[illegible]	[illegible]	[illegible]	[illegible]
RNA	25–100 ng/ml	Standard buffer + 50% formamide	Hybridize overnight at +50°C–68°C.
		DIG Easy Hyb	Hybridize overnight at +50°C–68°C.
DNA	25–100 ng/ml	High SDS buffer	Hybridize overnight at +37°C–50°C.
		DIG Easy Hyb	Hybridize overnight at +37°C–50°C.
Oligonucleotide tailed end labeled	0.1–2 pmol/ml 1–10 pmol/ml	Standard buffer	Hybridize for 1–6 h; hybridization temperature varies considerably and can be approximated by considering probe length and G plus C content. (To determine the T_m, add 4°C for each G or C and 2°C for each T or A). To prevent non-specific hybridization signals, hybridization with a tailed oligonucleotide should be performed with 0.1 mg/ml Poly(A) in the prehybridization and hybridization solutions. Additionally, 5 µg/ml Poly d(A) may be added to further blocking.

*The conditions given here are **stringent** conditions applicable if probe and target have 100% homology and a GC content of about 50%.

Table 9. Optimal hybridization conditions for different probe types.

2. Heat-denature the probe in a boiling water bath for 10 min.

   Oligonucleotide probes do not require denaturation prior to dilution unless extensive secondary structure is predicted from the sequence.
3. Dilute the probe in prehybridization solution. See Table 9 for recommended probe concentrations.
4. Discard the prehybridization solution from the bag, and add the hybridization solution containing the DIG-labeled probe. Allow the probe to hybridize. See Table 9 for recommended hybridization conditions.
5. At the end of the hybridization, pour the hybridization solution from the bag into a tube (with cap) that can withstand freezing and boiling (*e.g.*, a 50 ml polypropylene tube).

   This used hybridization solution contains unannealed DIG-labeled probe. The entire solution can be reused in future hybridization experiments. Store DIG-labeled DNA probes at –20°C; store DIG-labeled RNA probes at –70°C. DIG-labeled probes stored in this manner are stable for at least one year. For reuse, thaw and denature by heating to +68°C for 10 min.

❻ Wash the membrane twice, 15 min per wash, in 2X wash solution at room temperature.
These washes (steps 6 and 7) remove unbound probe, which would otherwise lead to high background.

❼ Wash the membrane twice, 15 min per wash, in 0.5X wash solution.
Wash long probes (>100 bp) at +68°C. For shorter probes, the washing temperature must be determined empirically.

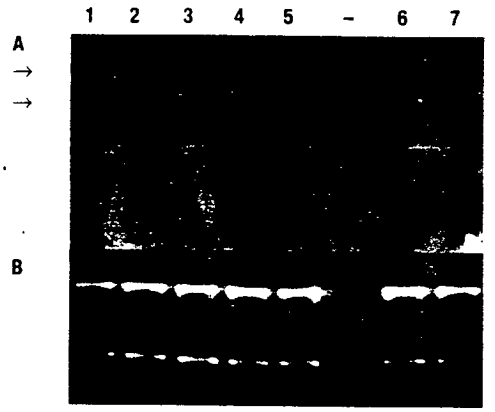

Figure 13. Example of a northern blot with a DIG-labeled RNA probe. Approximately 200 ng of total RNA from rat spinal cord (1), cortex (2), spleen (3), kidney (4), and liver (5,6,7) were run on a 1.5% agarose/ formaldehyde gel and transferred to a nylon membrane. Specific mRNA was detected with a 2.5 kb digoxigenin-labeled antisense RNA probe derived from zinc finger cDNA. For quantification, lanes 6 and 7 contain 0.1 pg and 1 pg, respectively, of a synthetic sense RNA derived from the same cDNA.

A. 45 min exposure of the membrane 2 h after the start of the detection reaction with the chemiluminescence substrate Lumigen PPD. A 4.8 kb mRNA is detected in all tissues. The amount of mRNA in liver approximately corresponds to the 0.1 pg standard in lane 6 running at 2.5 kb. Arrows indicate the positions of the 18S and 28S ribosomal RNAs.

B. Photograph of the Ethidium bromide-stained 18S and 28S RNAs after transfer to a nylon membrane.

Data were kindly provided by U. Pott, Brain Research Institute, Zurich, Switzerland.

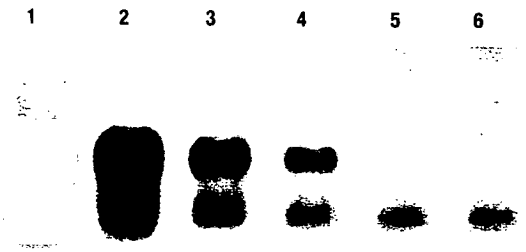

Figure 14. Comparison of extraction efficiency. RNA was extracted from different amounts of mouse heart tissue after the addition of 4 pg recovery standard.

Lane 1. Total RNA was extracted from 80 mg heart tissue by the acid guanidinium thiocyanate-phenol-chloroform method as described in reference 1. **Lanes 2 to 6:** mRNA was extracted as described in reference 2 from 80 mg (lane 2), 16 mg (lane 3), 8 mg (lane 4), 1.6 mg (lane 5), and 0.8 mg (lane 6) heart tissue. 0.8 mg of heart tissue contains only about 50 ng Poly(A)$^+$ RNA and less than 300 fg nerve growth factor (NGF) mRNA, which is below the detection limit of conventional northern blots. The RNA was glyoxylated, separated in 1.2% agarose gel, and transferred to a positively charged Nylon Membrane (Boehringer Mannheim). After hybridization with a digoxigenin-labeled cRNA probe, NGF mRNA was detected by Lumigen PPD chemiluminescent substrate. Hybridization and detection were performed under standard conditions described in DIG labeling and detection protocols from Boehringer Mannheim.

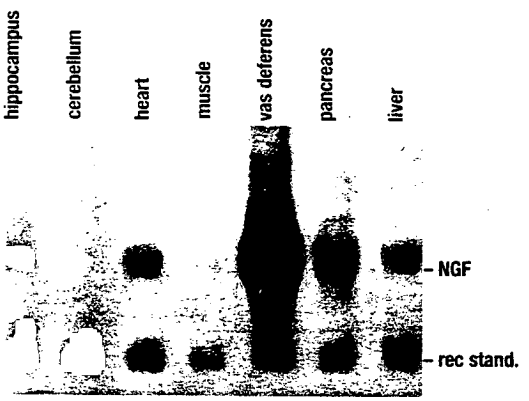

Figure 15. Extraction of mRNA from various rat tissues. mRNA was extracted from the indicated rat tissues (50 mg wet weight each) by the method described in reference 2. In order to determine the extraction efficiency, 8 pg of a shortened polyadenylated NGF recovery standard (reference 3) was added to each sample prior to mRNA extraction. Hybridization and detection were performed as described in Figure 14.

Data from Figures 14 and 15 were kindly provided by Dr. B. Hengerer, CIBA GEIGY AG, Basel, Switzerland.

WHAT TO DO NEXT *Proceed to the "Detection" division of this User's Guide, which begins on page 56.*

References

1. Chomcynski, P. and Sacchi, N. (1987) Single-step method for RNA isolation by acid guanidinium-thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156.
2. Hengerer, B. (1993) A rapid procedure for mRNA extraction from a large number of samples. *BioTechniques* **14**(4):522–524.
3. Heuman, R. and Thoenen, H. (1986) Comparison between the time course of changes in nerve growth factor NGF protein levels and those of its messenger RNA, in the cultured rat iris. *J. Biol. Chem.* **261**:9246.

RNA Dot Blotting

Dot blots and slot blots are rapid methods for the qualitative screening of RNA. The same hybridization and detection procedures used with northern blots are also performed on RNA dot blots; therefore, proceed to the northern blotting application (page 52) after completing this dot blotting procedure.

Required solutions

Solutions required for the hybridization and detection of RNA dot blots, but not specifically required by the dot blotting procedure given here, are listed in the northern blotting application (page 52).

Required solutions	Descriptions
DEPC-treated H_2O	Mix 0.1% dimethylpyrocarbonate with water, incubate for 30 min at room temperature, and autoclave.
RNA dilution buffer	Mix DEPC-treated H_2O:20X SSC:Formaldehyde (5:3:2)

Procedure

1. Dilute the RNA sample in RNA dilution buffer.
2. Mark the membrane lightly with a pencil to identify each dilution before spotting.
3. Using a micropipettor, spot 1 µl of the RNA sample onto a dry nylon membrane. Alternatively, the sample can be applied using a slot- or dot-blotting manifold.
4. Fix the RNA to the membrane by U.V. crosslinking or baking in an oven at +120°C for 30 min (or at 80°C for 1 h). With nitrocellulose membranes, use a vacuum oven at +80°C for 2 h.

WHAT TO DO NEXT *Hybridize the samples on the dot blot according to the recommendations described in the northern blotting application, which begins on page 52.*

Chemiluminescent Detection

For added convenience, we now offer three chemiluminescent substrates (Lumi-Phos 530, Lumi-Phos Plus, and Lumigen PPD), as well as the Genius 7 Luminescent Detection Kit, for chemiluminescent detection of DIG-labeled nucleic acids. While Lumi-Phos 530 and Lumigen PPD offer the same sensitivity when detection is performed on nylon membranes – both allow the detection of 0.03 pg of DIG-labeled DNA – Lumi-Phos 530 offers greater sensitivity in chemiluminescent ELISA procedures. Lumi-Phos Plus exhibits the same sensitivity as these other two substrates but can be used on nitrocellulose membranes; therefore, if you plan on performing chemiluminescent detection on a **nitrocellulose** membrane, you must use **Lumi-Phos Plus substrate**.

Chemiluminescent detection is a three-step process. In the first step, membranes are treated with Blocking reagent to prevent nonspecific attraction of antibody to the membrane. Then, membranes are incubated with a dilution of anti-digoxigenin Fab fragments, which are conjugated to alkaline phosphatase. In the third step, the membrane carrying the hybridized probe and bound antibody conjugate is reacted with one of the three chemiluminescent substrates and exposed to X-ray film to record the chemiluminescent signal.

Products required

Products and solutions required for chemiluminescent detection are listed below.

Chemiluminescent alkaline phosphatase substrates are available in one of four forms:

- The **Genius 7 Luminescent Detection Kit (Cat. No. 1363 514)** contains all of the reagents required for chemiluminescent detection of digoxigenin-labeled nucleic acids, including Lumigen PPD. It also contains a DIG-labeled control DNA for practicing chemiluminescent detection.
- **Lumi-Phos 530** can be purchased as a separate reagent and used to replace the colorimetric detection reagents (X-phosphate and NBT) in the **Genius 1 DNA Labeling and Detection Kit (Cat. No. 1093 657)** or the **Genius 3 Nucleic Acid Detection Kit (Cat. No. 1175 041)**.
- **Lumigen PPD** can also be purchased as a separate reagent and used to replace the colorimetric detection reagents (X-phosphate and NBT) in the **Genius 1 DNA Labeling and Detection Kit (Cat. No. 1093 657)** or the **Genius 3 Nucleic Acid Detection Kit (Cat. No. 1175 041)**.
- **Lumi-Phos Plus** can be purchased as a separate reagent and used to replace the colorimetric detection reagents (X-phosphate and NBT) in the **Genius 1 DNA Labeling and Detection Kit (Cat. No. 1093 657)** or the **Genius 3 Nucleic Acid Detection Kit (Cat. No. 1175 041)**.

	Description	
Anti-DIG-alkaline phosphatase	750 units/ml Anti-digoxigenin [Fab] conjugated to alkaline phosphatase	1. Vial 3, Genius 7 Kit 2. Vial 8, Genius 1 Kit 3. Vial 3, Genius 3 Kit 4. Cat. No. 1093 274
One of the following:		
Lumi-Phos 530 store at +4°C, protected from direct light; warm to room temperature (r.t.) before using	0.33 mM Lumigen PPD [4-methoxy-4-(3-phosphatephenyl)-spiro-(1,2-dioxetane-3,2'-adamantane) disodium salt]; 750 mM 2-amino-2-methyl-1-propanol buffer (pH 9.6); 0.88 mM $MgCl_2$; 1.13 mM cetyltrimethyl-ammonium bromide; 0.035 mM fluorescein surfactant	Cat. Nos. 1413 155, 1275 470, 1413 163
OR		
Lumigen PPD store at +4°C, protected from direct light; warm to r.t. and dilute before using	10 mg/ml (23.5 mM) Lumigen PPD [4-methoxy-4-(3-phosphatephenyl)-spiro-(1,2-dioxetane-3,2'-adamantane) disodium salt]	1. Vial 5, Genius 7 Kit 2. Cat. No. 1357 328
OR		
Lumi-Phos Plus store at +4°C, protected from light; warm to r.t before using	0.33 mM Lumigen PPD [4-methoxy-4-(3-phosphatephenyl)-spiro-(1,2-dioxetane-3,2'-adamantane) disodium salt], 200 mM 2-amino-2-methyl-1-propanol buffer (pH 9.6), 0.88 mM $MgCl_2$, and a proprietary enhancer.	1. Cat. Nos. 1581 082, 1581 104

Additionally required solutions

Except TE buffer, all of the following required solutions are available in a ready-to-use form in the **DIG Wash and Block Buffer Set (Cat. No. 1585 762)**. Bottle numbers for this set are given in parentheses. Alternatively, they can be prepared from separate reagents according to procedures described in Appendix B.

Additionally required reagent	Description
Washing buffer (Bottle 1; dilute 1:10 with H_2O)	100 mM maleic acid, 150 mM NaCl; pH 7.5 (+20°C); 3% (v/v) Tween 20
Maleic acid buffer (Bottle 2; dilute 1:10 with H_2O)	100 mM maleic acid, 150 mM NaCl; pH 7.5 (+20°C)
Blocking solution (Bottle 3; dilute 1:10 with 1X Maleic acid buffer)	1% (w/v) Blocking reagent for nucleic acid hybridization dissolved in Maleic acid buffer If necessary, treat with DEPC to destroy RNases. Autoclave the solution, and store at room temperature, +4°C, or –20°C. Blocking solution is cloudy and should not be filtered. It is stable for at least two weeks when stored at +4°C, but must then be brought to room temperature before use.
Detection buffer (Bottle 4; dilute 1:10 with H_2O)	100 mM Tris-HCl, 100 mM NaCl; pH 9.5 (+20°C)
TE buffer	10 mM Tris, 1 mM EDTA; pH 8.0 (+20°C)
DEPC-treated H_2O (for RNA probes only)	Sterile, distilled water treated with 0.1% diethylpyrocarbonate (DEPC)

Guidelines for handling Lumi-Phos 530, Lumi-Phos Plus, or Lumigen PPD

To maintain full activity, as well as the nuclease-, phosphatase-, and bacteria-free environment in which the chemiluminescent substrates Lumi-Phos 530, Lumi-Phos Plus, and Lumigen PPD are provided, adhere to the following precautions:

- Do not freeze the substrate.
- Do not place any non-sterile instrument (*e.g.*, pipet tips) into the substrate solutions.
- Remove the substrate from the bottle by pouring it into a sterile container using sterile technique or by transferring it with sterile pipettes. Wear powder-free gloves, and avoid touching the mouth of the bottle to anything.
- Dilution or re-use of Lumi-Phos 530 or Lumi-Phos Plus is not recommended, as contamination of the stock solution is likely.

To minimize background staining, adhere to the following precautions:

- Avoid touching the membrane with fingers (gloved or ungloved).
- Use blunt-ended forceps that have been washed and autoclaved (to avoid alkaline phosphatase contamination) to pick up membranes, and handle membranes only at their edges.
- Wear unpowdered gloves, and use hybridization bags free of dust and powder. Gloves or bags can be washed in distilled water before use.

Procedure

Perform all incubations at room temperature.

Incubations can be performed in a sealed hybridization bag or clean plastic tray. If a bag is used, remove all large air bubbles that may be present in the bag. If a tray is used, agitate the tray gently to ensure that the membrane is always covered. If you are using more than one membrane, add enough solution to cover all membranes.

1. After hybridization and post-hybridization washes, equilibrate the membrane in Washing buffer for 1 minute.
2. Allow the chemiluminescent substrate to come to room temperature.
3. Using a freshly washed dish or bag, block the membrane by gently agitating it in Blocking solution for 30–60 minutes. Near the end of the blocking period, prepare the antibody solution as described in step 4. Longer blocking times are acceptable.
4. Dilute the Anti-DIG-alkaline phosphatase 1:10,000 in Blocking solution. Mix gently by inversion. For example, for a 1:10,000 dilution, add 3 µl Anti-DIG-alkaline phosphatase to 30 ml Blocking solution and mix. This working antibody solution is stable for about 12 hours at +4°C.
5. Pour off the Blocking solution and incubate the membrane for 30 minutes in the antibody solution prepared in step 4.
6. Discard the antibody solution. Gently wash the membrane twice, 15 minutes per wash, in Washing buffer.

❼ Pour off Washing buffer and equilibrate the membrane in Detection buffer for two minutes. If using Lumigen PPD, dilute it 1:100 in Detection buffer. **Lumi-Phos 530 and Lumi-Phos Plus are pre-diluted; do not dilute!** It is important that the filter be kept wet before the chemiluminescent substrate is applied. If the membrane is even slightly dry, high backgrounds can occur.

❽ There are two methods of applying Lumi-Phos 530, Lumi-Phos Plus, or diluted Lumigen PPD. The single-filter method should be used when DNA is to be visualized on a single membrane. The filter-batching method is recommended for multiple membranes but may also be used when visualization is performed on a single membrane.

Single-filter method

a. Place the membrane between two sheets of acetate (plastic page protectors). Gently lift the top sheet of plastic and, with a sterile pipet, add approximately 0.5 ml (per 100 cm^2) of the chemiluminescent substrate in a line parallel to the side (closest to the fold of the page protector) of the membrane. Slowly lower the top sheet of plastic, allowing the substrate to spread evenly over the entire surface of the membrane. Add additional substrate if the membrane is not completely covered. With a damp lab tissue (*e.g.*, Kimwipe®), gently wipe the top sheet to remove any bubbles present under the sheet and to create a liquid seal around the membrane.
. Proceed to step 9.

Filter-batching method

a. Pipette 5–10 ml of the appropriate chemiluminescent substrate into the center of sterile dish. Using blunt-end forceps, place the membrane in the dish. Tilt the dish until the membrane is thoroughly saturated.
b. Remove the membrane from the substrate, and allow any excess liquid to drip off. **Do not allow the membrane to dry.**
c. Cover the damp membrane by placing it between two clear acetate sheets or page protectors.
d. Wipe the top sheet with a damp lab tissue (*e.g.*, Kimwipe®) to remove any bubbles present between the sheet and the membrane.
e. Repeat the filter-batching method until the chemiluminescent substrate has been applied to all membranes. To prevent the membrane from drying out, avoid repeated exposure to air. After treating the final membrane, proceed to step 9.

❾ For the briefest exposure to X-ray film, the alkaline phosphatase chemiluminescent reaction must be at a steady state. At room temperature, 7–8 h are required to reach a steady state reaction. Once a steady state is reached, single-copy gene detection on a human genomic blot can be obtained with an approximate exposure time of 15 min. If the membrane is exposed before the steady state is reached, approximately 60 min of exposure is required for single-copy gene detection on a human genomic blot. **Therefore, to shorten exposure times, we recommend incubation of the membrane for 15 min at +37°C directly after addition of the chemiluminescent substrate and before exposure to X-ray film.**

❿ For detection of the chemiluminescent signal, the membrane is exposed to standard X-ray film (*e.g.*, Kodak . XAR or Chronex 8 from Dupont). Multiple exposures from a single blot can be obtained for up to 2 days after the addition of the chemiluminescent substrate (Figure 16).

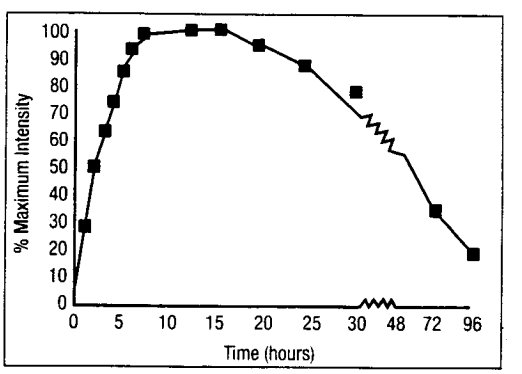

Figure 16. Time course of Lumi-Phos 530 light emission on a nylon membrane. Light intensity increases for 7–8 hours, is maximal for 12 hours, and then slowly decreases. In a Southern blot of 10 µg of human DNA, a single-copy gene can be detected in 1 hour when the X-ray film exposure is performed immediately after the application of Lumi-Phos 530. The same gene can be detected in as little as 15 minutes if the X-ray film exposure is performed 8–24 hours after application of the Lumi-Phos 530 or if the membrane is preincubated for 15 min at +37°C directly after addition of Lumi-Phos 530 or Lumigen PPD.

Kimwipe® is a registered trademark of Kimberly-Clark Corporation, Roswell, GA.

Colorimetric Detection with NBT and X-Phosphate

With the Genius System, detection can be performed with the colorimetric detection reagents NBT and X-Phosphate (BCIP).

Products required

Products and solutions required for colorimetric detection are listed below. The colorimetric detection reagents are available separately, in the **Genius 1 DNA Labeling and Detection Kit (Cat. No. 1093 657)**, or in the **Genius 3 Nucleic Acid Detection Kit (Cat. No. 1175 041)**.

Name in procedure	Description	Available as
Anti-DIG-alkaline phosphatase	750 units/ml Anti-digoxigenin [Fab] conjugated to alkaline phosphatase	1. Vial 8, Genius 1 Kit 2. Vial 3, Genius 3 Kit 3. Cat. No. 1093 274
NBT solution	75 mg/ml nitroblue tetrazolium salt in 70% (v/v) dimethylformamide	1. Vial 9, Genius 1 Kit 2. Vial 4, Genius 3 Kit 3. Cat. No. 1383 213 (sold as 100 mg/ml; dilute prior to use)
X-phosphate solution	50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate), toluidinium salt in 100% dimethylformamide	1. Vial 10, Genius 1 Kit 2. Vial 5, Genius 3 Kit 3. Cat. No. 1383 221

Additionally required solutions

Except TE buffer, all of the following required solutions are available in a ready-to-use form in the **DIG Wash and Block Buffer Set (Cat. No. 1585 762)**. Bottle numbers for this set are indicated in parentheses. Alternatively, they can be prepared from separate reagents according to procedures described in Appendix B.

Additionally required solution	Description
Washing buffer (Bottle 1; dilute 1:10 with H_2O)	100 mM maleic acid, 150 mM NaCl; pH 7.5 (+20°C); 3% (v/v) Tween 20
Maleic acid buffer (Bottle 2; dilute 1:10 with H_2O)	100 mM maleic acid, 150 mM NaCl; pH 7.5 (+20°C)
Blocking solution (Bottle 3; dilute 1:10 with 1X Maleic acid buffer)	1% (w/v) Blocking reagent for nucleic acid hybridization dissolved in Maleic acid buffer If necessary, treat with DEPC to destroy RNases. Autoclave the solution, and store at room temperature, +4°C, or –20°C. Blocking solution is cloudy and should not be filtered. It is stable for at least two weeks when stored at +4°C, but must then be brought to room temperature before use.
Detection buffer (Bottle 4; dilute 1:10 with H_2O)	100 mM Tris-HCl, 100 mM NaCl; pH 9.5 (+20°C) To increase the speed of colorimetric detection, 50 mM $MgCl_2$ may be added to the Detection buffer; however, filter the $MgCl_2$-containing buffer through a 0.45 µm membrane filter before use. This eliminates any precipitated $MgCl_2$, which can contribute to background if not removed.
TE buffer	10 mM Tris, 1 mM EDTA; pH 8.0 (+20°C)
DEPC-treated H_2O (for RNA probes only)	Sterile, distilled water treated with 0.1% diethylpyrocarbonate (DEPC)

Procedure

Perform all incubations at room temperature.

❶ After hybridization and post-hybridization washes, equilibrate the membrane in filtered Washing buffer for 1 minute.

❷ Using a freshly washed dish or bag, block the membrane by gently agitating it in Blocking solution for 30–60 minutes. Near the end of the blocking period, prepare the antibody solution as described in step 3.

Longer blocking times are also acceptable.

For sufficient blocking, there must be ample room in the bag or dish to allow for unrestricted shaking of the membrane. If you are using more than one membrane, add enough solution to cover all membranes.

❸ Dilute the Anti-DIG-alkaline phosphatase 1:5,000 in Blocking buffer for a working concentration of 150 mU/ml. Mix gently by inversion. For example, add 6 µl Anti-DIG-alkaline phosphatase to 30 ml Blocking solution, and mix.

This working antibody solution is stable for about 12 hours at +4°C.

4. Pour off the Blocking solution, and incubate the membrane for 30 minutes in the antibody solution prepared in step 3. If a bag is used, remove all large air bubbles that may be present in the bag. If a tray is used, agitate the tray gently to ensure that the membrane is always covered.
5. Discard the antibody and Blocking solution. Wash twice, 15 minutes per wash, in 100 ml Washing buffer. These washes remove unbound antibody.
6. Mix 45 µl NBT solution and 35 µl X-phosphate solution in 10 ml of Detection buffer.
   This freshly prepared Color Substrate Solution will be used in step 8. Protect from direct light before use.
7. Equilibrate the membrane in 20 ml Detection buffer for 2 minutes.
8. Pour off the Detection buffer, and add approximately 10 ml Color Substrate Solution to the membrane. Incubate the membrane in a sealed plastic bag or box in the dark. Do not shake the container while the color is developing.
   The membrane can be exposed to light for short periods to monitor the color development. The color precipitate starts to form within a few minutes, and the reaction is usually complete after 12 hours. **Do not shake.**
9. Once the desired spots or bands are detected, wash the membrane with H_2O to prevent over-development. If the membrane is to be reused, use sterile H_2O or a sterile buffer (*e.g.*, TE buffer) to stop the development.

Results can be documented by photocopying the wet filter or by photography. Photocopying onto overhead transparencies allows for densitometric scanning; to do this, the color reaction can be interrupted for a short time and continued afterwards.

The membrane can also be dried at room temperature or by baking at +80°C, and then stored, although the color fades upon drying. To revitalize the color, wet the membrane in TE buffer. **If the membrane is to be reprobed, do not allow the membrane to dry.**

Alternatively, store the membrane in a sealed plastic bag containing TE buffer. In this case, the color remains unchanged.

Multicolor Detection

Detection of digoxigenin-, biotin-, and fluorescein-labeled nucleic acids can be performed with successive enzyme immunoassays that yield three different colors.

The multiple-labeling and multicolor detection scheme allows discrete nucleic acid sequences to be detected with differently colored hybridization signals on the same blot. Nucleic acid probes labeled with digoxigenin, fluorescein, or biotin are hybridized simultaneously to immobilized target nucleic acids. The labels are detected by alkaline phosphatase conjugates (Anti-digoxigenin-alkaline phosphatase, Anti-fluorescein-alkaline phosphatase, or Streptavidin-alkaline phosphatase) and three different naphthol-AS-phosphate/diazonium salt combinations as substrates for alkaline phosphatase. The detection reactions are carried out consecutively, with a heat/EDTA treatment between each to inactivate the formerly bound alkaline phosphatase. The resulting hybridization signals are green, red, or blue for targets that have homology to only one probe. If the target DNA or RNA fragment is homologous to more than one of the probes, the resulting signal is a mixed color.

This method is of advantage in all applications where different hybrids are to be detected on the same blot or specimen. Useful applications include genomic Southern blots of lower eukaryotes, plasmid mapping, northern blots comparing the abundance of different mRNAs, and colony and plaque hybridizations.

Multicolor detection allows 0.3 pg of homologous DNA to be detected within 2 h; this sensitivity is satisfactory for single-copy gene detection in genomic blots of lower eukaryotes like yeast or *Drosophila*, but can sometimes be insufficient for genomic blots of mammalian DNA. When greater sensitivity is required, use the colorimetric BCIP/NBT substrate or the chemiluminescent substrates Lumigen PPD, Lumi-Phos 530, or Lumi-Phos Plus.

Products required

Products and solutions required for multicolor detection are listed below. Refer to Appendix B for details on preparing the additionally required solutions.

Name in procedure	Description	Available as
Anti-digoxigenin-alkaline phosphatase	Anti-digoxigenin [Fab] conjugated to alkaline phosphatase 2. 3.	1. Vial 8, Genius 1 Kit Vial 3, Genius 3 Kit Cat. No. 1093 274
Anti-fluorescein-alkaline phosphatase	Anti-fluorescein [Fab] conjugated to alkaline phosphatase	Cat. No. 1426 388
Streptavidin-alkaline phosphatase	Streptavidin conjugated to alkaline phosphatase	Cat. No. 1093 266
Multicolor Detection Set	Alkaline phosphatase substrate tablets for 3 x 50 detection reactions (for the detection of 60 blots of 10 x 10 cm^2 with three colors) The Set contains: 1. "Green" AP substrate tablets; 50 tablets, each containing 2 mg of naphthol-AS-Gr-phosphate and 3.5 mg of Fast Blue B 2. "Red" AP substrate tablets; 50 tablets, each containing 2 mg of naphthol-AS-phosphate and 1 mg of Fast Red TR 3. "Blue" AP substrate tablets; 50 tablets, each containing 2 mg of naphthol-AS-phosphate and 3.5 mg of Fast Blue B	Cat. No. 1465 341
Blocking reagent	Blocking reagent for nucleic acid hybridization	Cat. No. 1096 176

Additionally required solutions

In addition, you will need to prepare the following solutions. See Appendix B for solution preparation.

Additionally required solutions	Description
EDTA	50 mM EDTA, pH 8.0
Maleic acid buffer	0.1 M maleic acid, 0.15 M NaCl, pH 7.5 (+20°C) ; adjusted with solid or concentrate NaOH; autoclaved
Washing buffer	100 mM Maleic acid, 150 mM NaCl; pH 7.5 (+20°C); 3% Tween 20
TE buffer	10 mM Tris, 1 mM EDTA; pH 8.0 (+20°C)
Blocking reagent stock solution	Blocking reagent for nucleic acid hybridization dissolved in Maleic acid buffer to a final concentration of 10% (w/v); afterwards, the solution is autoclaved and stored at +4°C or –20°C
Blocking solution for DIG- and fluorescein-labeled probes	Sterile Blocking reagent stock solution diluted 1:10 in Maleic acid buffer (final concentration = 1% Blocking Reagent)
Blocking solution for biotin-labeled probes	Sterile Blocking reagent stock solution diluted 1:2 in Maleic acid buffer (final concentration = 5% Blocking Reagent)
Antibody solutions	Anti-digoxigenin-alkaline phosphatase, anti-fluorescein-alkaline phosphatase, or streptavidin-alkaline phosphatase, each 150 mU/ml in the appropriate blocking solution
Detection buffer	0.1 M Tris-HCl, pH 9.5 (+20°C); 0.1 M NaCl
Color substrate working solutions (freshly diluted in Detection buffer)	Prepare fresh working solution each day. Dissolve one tablet in 10 ml Detection buffer while stirring at room temperature. The "Red" and "Blue" tablets dissolve completely within a few minutes. The "Green" tablets do not dissolve completely, but this does not influence the results. **Note:** We recommend that gloves be worn when handling the color substrate solutions.

Labeling with Fluorescein or Biotin

As with DIG labeling, nucleic acid probes can be labeled with fluorescein or biotin by random-primed DNA labeling, DNA amplification by PCR, *in vitro* RNA transcription, or oligonucleotide end-labeling or tailing. The labels can also be introduced into oligonucleotides via chemical synthesis. Fluorescein- and biotin-labeled probes are most conveniently prepared with the High Prime Fluorescein DNA Labeling Mix (Cat. No. 1585 622) and High Prime Biotin DNA Labeling Mix (Cat. No. 1585 649). Fluorescein-12-dUTP and Biotin-16-dUTP are also available as single reagents, and can replace DIG-11-dUTP in the Genius Kit protocols. Labeling protocols for these nucleotide analogs are given in the respective pack inserts. The labeling reactions can also be carried out without kits using single reagents; a listing of the single reagents for nonradioactive labeling is given in Appendix C.

Hybridization

The differently labeled probes can be hybridized simultaneously to a blot or *in situ* to homologous sequences according to the protocols given for hybridization of DIG-labeled probes.

It is especially important that the probe concentration for hybridization be optimized for all three probes. We strongly recommend a mock hybridization on a naked piece of membrane or a Dot Blot for this evaluation. A protocol for this is given on page 42.

If multiple (multicolor) detections are performed on the same membrane, the hybrids must be fixed to the membrane after the stringency washes (but before detection). This is necessary because heat treatment is used to inactivate the alkaline phosphatase between detections. To crosslink the hybrids, expose the membrane to UV light for 3 min at 254 nm. If only one label and one color has been used, fixation of the hybrids is not necessary.

Multicolor Detection

Detection of the different labels is performed by binding the respective antibody- or streptavidin-alkaline phosphatase conjugate. A different combination of naphthol-phosphate and diazonium salt is used to yield a different color for each conjugate (green, blue, or red).

The detection reactions are performed consecutively, with heat-inactivation of the alkaline phosphatase between the detections. As stated above, hybrids must be stabilized by UV-crosslinking (3 min at 254 nm) if different labels are detected consecutively.

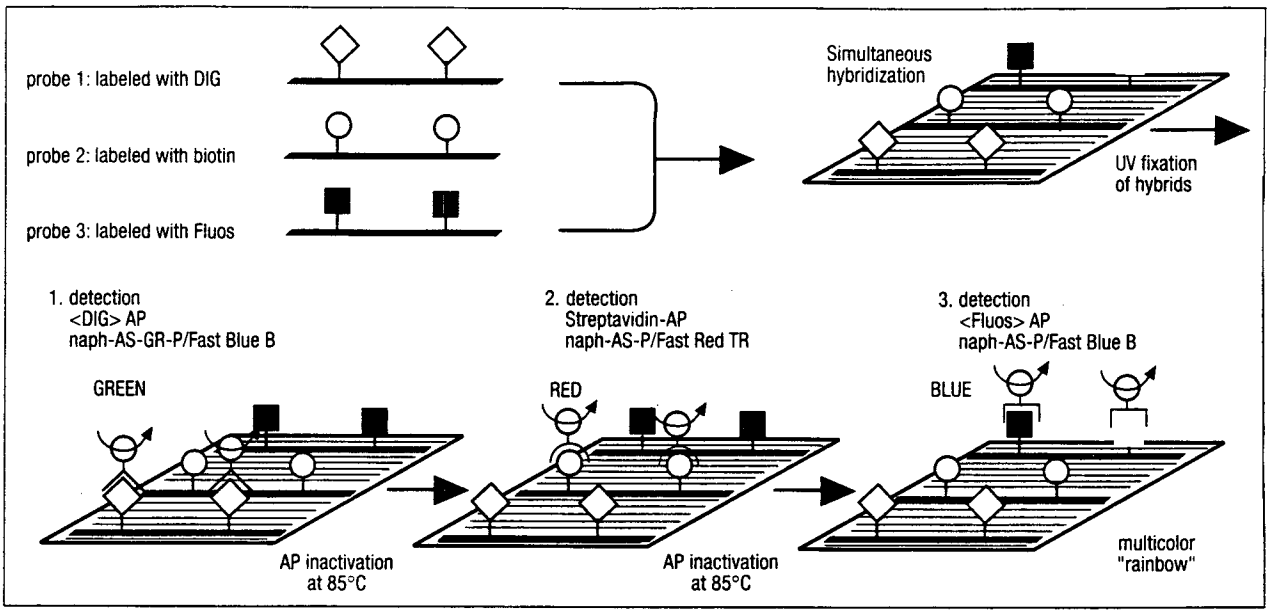

Figure 17. Principle of the Multicolor Detection Set.

The following procedure describes triple detection; orders of detection and colors may be changed according to individual requirements.

All incubations are performed at room temperature and, except for the color reaction, with shaking or mixing. The volumes of the solutions are calculated for a membrane size of 100 cm^2, and should be adjusted to fit other membrane sizes. Blocking and equilibration steps may proceed for longer periods if more convenient.

Detection of DIG- or Fluorescein-labeled Hybrids

Procedure

1. If multicolor detection is to be performed, fix hybrids by UV exposure (3 min at 254 nm) or baking. This should be performed after hybridization and stringency washes.
2. Wash the membrane briefly in Washing buffer.
3. Incubate for at least 30 min with about 100 ml of Blocking solution for DIG- and fluorescein-labeled probes (1% [w/v]).
4. Dilute the anti-digoxigenin-alkaline phosphatase or anti-fluorescein-alkaline phosphatase 1:5,000 in Blocking solution for DIG- and fluorescein-labeled probes (final concentration, 150 mU/ml).
   These diluted antibody-conjugate solutions are stable for about 12 h at +4°C.
5. Incubate the membrane for 30 min in about 20 ml of the diluted antibody conjugate solution prepared in step 4.
6. Wash twice,15 min per wash, with 100 ml of Washing buffer.
   These washes remove unbound antibody conjugate.
7. Equilibrate the membrane for at least 2 min in 20 ml Detection buffer.
8. Dissolve one substrate tablet in 10 ml Detection buffer. Incubate the membrane with 10 ml of one of the color substrate solutions (freshly prepared) for about 45 min, then replace with fresh color substrate solution if necessary.
   The colored precipitate begins to form within a few minutes and can be allowed to proceed for up to 2 h (until the desired signal intensity is obtained).
9. Inactivate the alkaline phosphatase as described below or stop the final color reaction by washing the membrane with TE buffer.

Alkaline Phosphatase Inactivation (to be performed between ... and ... if desired)

1. Wash the membrane briefly in TE buffer.
2. Incubate the membrane for at least 10 min at +85°C in EDTA.
3. Wash the membrane twice for 5 min in Washing buffer.
   This wash removes the EDTA.
4. Proceed to the next detection procedure.

Detection of Biotin-labeled Hybrids

1. If multicolor detection is to be performed, fix hybrids by UV exposure (3 min at 254 nm) or baking.
   This should be performed after hybridization and stringency washes.
2. Wash the membrane briefly in Washing buffer.
3. Incubate the membrane for at least 30 min in about 100 ml Blocking solution for biotin-labeled probes (5% [w/v]).
4. Dilute Streptavidin-alkaline phosphatase 1:5000 in Blocking solution for biotin-labeled probes (final concentration, 150 mU/ml).
5. Incubate the membrane for 30 min in about 20 ml diluted streptavidin-conjugate solution prepared in step 4.
   Diluted streptavidin-conjugate solutions are stable for about 12 h at +4°C.
6. Wash twice, for 15 min per wash, in 100 ml of Washing buffer.
   These washes remove unbound conjugate.
7. Equilibrate the membrane for at least 2 min in 20 ml Detection buffer.
8. Dissolve one tablet in 10 ml Detection buffer. Incubate the membrane with 10 ml freshly prepared color substrate solution for about 45 min, then replace with fresh color substrate solution if necessary.
   The colored precipitate begins to form within a few minutes and can be allowed to proceed for up to 2 h (until the desired signal intensity is obtained).
9. Inactivate the alkaline phosphatase as described above or stop the final color reaction by washing the membrane with TE buffer.

Results can be documented by photography. The colors do not fade when the membranes are dried and stored at room temperature.

Stripping and Reprobing of Membranes

Membranes can only be reused if only one label and color has been used and if the hybrids have not been UV-crosslinked or baked. Do not allow the membrane to dry out if it is to be reprobed.

Removal of color precipitate

The color precipitate can be removed by washing the membrane with ethanol (red: room temperature; blue and green: +50° to 65°C; put the membrane and ethanol in a sealed plastic bag in a hybridization oven or waterbath). Replace the solution from time to time until the precipitate is completely dissolved.

Removal of the probe

1. Thoroughly rinse the membrane in water.
2. Incubate twice for 20 min in 0.2 M NaOH, 0.1% (w/v) SDS at +37°C.
3. Rinse the membrane in 2X SSC. The membrane may now be dried or used directly for hybridization.
   Alternatively, any established procedure for removing hybridized probes (e.g., heating in SDS buffer or formamide-containing buffers at neutral pH) can be used.

Reference

1. Holtke, H.J., Ettl, I., Finken, M., West, S. and Kunz, W. (1992) "Multiple Nucleic Acid Labeling and Rainbow Detection." *Anal. Biochem.* **207**:24-31.

Stripping Membranes for Reprobing

Only Nylon Membranes can be stripped and reprobed because

- The reagents required to remove color dissolve nitrocellulose membranes.
- The method of stripping membranes for reprobing depends on the method of detection (colorimetric or chemiluminescent) that has been used.

Required solutions

Additionally required reagents	Description
Dimethylformamide	100% ACS grade N,N-dimethylformamide (DMF)
H_2O	Sterile, distilled water
Alkaline probe-stripping solution	0.4 N NaOH, 0.1% SDS
2X SSC buffer	300 mM NaCl, 30 mM sodium citrate
Proteinase K	1 mg/ml Proteinase K, 0.2% (w/v) SDS
2X SSC/0.1% SDS	300 mM NaCl, 30 mM sodium citrate, 0.1% (w/v) SDS
Southern probe-stripping solution	50% formamide; 10 mM $NaPO_4$, pH 6.5
Northern probe-stripping solution	60% formamide; 50 mM Tris-HCl, pH 8; 1% (w/v) SDS
DEPC-treated H_2O	Sterile, distilled water treated with 0.1% diethylpyrocarbonate (DEPC)

Procedure for removing the color precipitate

1. Using a water bath, heat a large glass beaker of dimethylformamide to +50–60°C.
   Caution: Dimethylformamide is volatile and flammable. Keep away from sparks and open flames. Work in a fume hood. The flash point of dimethylformamide is at +67°C.
2. Incubate the membranes in the heated dimethylformamide until the blue color has been removed. Changing the dimethylformamide solution frequently will increase the speed of decolorization.
3. Rinse the membranes thoroughly in H_2O.
4. Proceed to probe removal.
   CAUTION: Do not allow the membrane to dry prior to probe removal.

Procedure for removing the chemiluminescent substrate

1. Wash the membrane in H_2O for 1 min.
2. Proceed to probe removal.
   CAUTION: Do not allow the membrane to dry prior to probe removal.

Procedures for removing the probe from Southern, DNA Dot, and colony/plaque hybridizations

Method I (Recommended for Alkali-labile DIG-labeled probes)

1. Wash the membranes in H_2O for 1 min.
2. Incubate the membranes twice for 10 min Alkaline probe-stripping solution at +37°C. This incubation removes the alkali-labile DIG-labeled probe.
3. Rinse the membranes thoroughly in 2X SSC.
4. Commence reprobing with the prehybridization step of the desired hybridization procedure.

Method II (Not recommended for probes labeled with alkali-labile DIG-11-dUTP)

When several reprobing experiments are to be carried out, the method of Dubitsky *et al.* (*BioTechniques*, 1992) is recommended:

1. Wash twice, 30 min per wash, in 0.5–1 mg/ml Proteinase K, 0.1–0.2% SDS at +68°C.
2. Wash twice, 5 min per wash, in 2X SSC/0.1% SDS.
3. Wash twice, 30 min per wash, in Southern probe-stripping solution at +68°C.
4. Rinse membranes briefly in 2X SSC.
5. Commence reprobing with the prehybridization step of the desired hybridization procedure.

Procedures for removing the probe from northern blots

Method I

1. Rinse the membrane thoroughly in sterile H_2O.
2. Incubate the membrane twice, 30 min per incubation, in Northern probe-stripping solution at +68°C.
3. Rinse the membrane, first in water, then in 2X SSC.
4. Commence reprobing with the prehybridization step of the desired hybridization procedure.

Method II (Best method for stripping northern blots probed with RNA)

1. Rinse the membrane in sterile H_2O.
2. Place the membrane in hybridization bag with DEPC-treated H_2O, 0.1% SDS; boil for 10 min.
3. Rinse the membranes in 2X SSC.
4. Commence reprobing with the prehybridization step of the desired hybridization procedure.

Note:

- After stripping, start with the prehybridization or store the filter wet in 2X SSC in a sealed plastic bag.
- Rewarm the stripping solution to the appropriate incubation temperature.
- For incubation, use a shaking waterbath or a hybridization oven.

The Genius Nonradioactive DNA Sequencing Kit

The Genius Nonradioactive DNA Sequencing Kit enables the convenient sequencing of DNA cloned into M13 or pUC vectors. The method is based upon the chain termination principle of Sanger, using primers and *Taq* DNA Polymerase for extension. Cloned DNA can be sequenced from the (+) single-stranded form of a M13 phage or a supercoiled pUC plasmid with the provided M13/pUC sequencing and reverse sequencing primers. With slight modifications, the kit may be used to sequence DNA from any vector (see below). The supplied primers are 5'-end labeled with digoxigenin and thus, following the extension/chain termination reaction, the separated and blotted DNA can be easily detected with alkaline phosphatase-labeled anti-digoxigenin antibody.

This nonradioactive method of DNA sequencing can be used for all sequencing applications. This convenient kit employs *Taq* DNA Polymerase for the extension reaction, which allows the use of high temperatures for sequencing. This reduces the likelihood of sequencing artifacts, especially where extensive secondary structures are probable. Sequences of at least 250 nucleotides can be obtained from single- or double-stranded DNA with chemiluminescent or colorimetric detection.

Primers for Extension

Supplied in the kit are the M13/pUC sequencing primer, which is complementary to the (+) strand sequence just 3' to the polylinker, and the M13/pUC reverse sequencing primer, which is homologous to 17 nucleotides immediately 5' to the polylinker (see Figure 18). Both primers are 5'-labeled with digoxigenin. For the (+) single-stranded form of a M13 phage vector, the primer anneals the 3' portion of the polylinker, and DNA synthesis proceeds toward the DNA of interest in the polylinker region. In double-stranded DNA sequencing, the DNA is first denatured, and both primers are annealed (in separate reactions) so that the DNA insert can be sequenced from both ends. Although the kit is directly applicable to sequencing DNA cloned into an M13 or pUC vector, it can be easily adapted to sequence DNA from any other vector that allows X-gal-based blue/white selection. See Appendix C for alternative DIG-labeled sequencing primers. Or if other primers are needed, these can be conveniently labeled at the amino-substituted 5'-end with Digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide Ester (details are provided in the package insert of the Genius 8 Oligonucleotide 5'-End Labeling Set).

Chain Elongation/Termination Reactions

Taq DNA Polymerase is employed to extend the annealed primers in four discrete reactions. Each reaction occurs in the presence of all four deoxyribonucleoside triphosphates and only one dideoxyribonucleoside triphosphate (ddATP, ddCTP, ddGTP, or ddTTP). Base-specific termination is thus achieved at sites where a dideoxynucleotide is incorporated. 7-Deaza-dGTP is provided and can be used as a substitute for dGTP to inhibit sequencing artifacts such as "band compression" produced by secondary structures in CG-rich regions of the newly synthesized DNA. Use of 7-Deaza-dGTP also leads to increased uniformity and intensity of the bands.

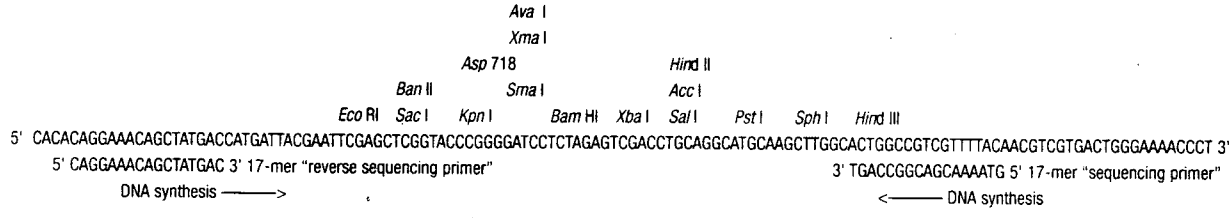

Figure 18. M13/pUC polylinker sequence (–) strand and sequencing primers.

Products required

The Genius Nonradioactaive DNA Sequencing Kit (Cat. No. 1449 443) contains reagents needed for nonradioactive DNA sequencing.

Description		Available
Control DNA, double-stranded	Double-stranded template plasmid DNA (pUC18 DNA) 0.25 µg/µl in Tris-EDTA buffer (TE buffer); pH 8	Vial 1
Control DNA, single-stranded	Single-stranded template DNA (M13mp18 DNA) 0.2 µg/µl in TE buffer pH 8	Vial 2
M13/pUC sequencing primer	M13/pUC 17-mer sequencing primer with the 5'-end labeled with digoxigenin; 1 pmol/µl in water	Vial 3
M13/pUC reverse sequencing primer	M13/pUC 17-mer reverse sequencing primer with the 5'-end labeled with digoxigenin; 1 pmol/µl in water	Vial 4
Reaction buffer	Buffer for the hybridization and chain elongation reaction	Vial 5
Taq DNA Polymerase	*Taq* DNA Polymerase, 3 units/µl for the chain elongation reaction	Vial 6
Extension/termination mixture ddATP (with dGTP, green)	Mixture containing dATP, dCTP, dGTP, dTTP, (25 µM each); and 950 µM $MgCl_2$; pH 7.5; 850 µM ddATP for termination	Vial 7
Extension/termination mixture ddCTP (with dGTP, green)	Mixture containing dATP, dCTP, dGTP, dTTP, (25 µM each); and 400 µM ddCTP for termination; 500 µM $MgCl_2$, pH 7.5	Vial 8
Extension/termination mixture ddGTP (with dGTP, green)	Mixture containing dATP, dCTP, dGTP, dTTP, (25 µM each); and 75 µM ddGTP for termination; 175 µM $MgCl_2$; pH 7.5	Vial 9
Extension/termination mixture ddTTP (with dGTP, green)	Mixture containing dATP, dCTP, dGTP, dTTP, (25 µM each); and 1275 µM ddTTP for termination; 1370 µM $MgCl_2$; pH 7.5	Vial 10
Extension/termination mixture ddATP (with 7-Deaza-dGTP, pink)	Mixture containing dATP, dCTP, 7-Deaza-dGTP, dTTP (25 µM each); and 850 µM ddATP for termination; 950 µM $MgCl_2$; pH 7.5	Vial 11
Extension/termination mixture ddCTP (with 7-Deaza-dGTP, pink)	Mixture containing dATP, dCTP, 7-Deaza-dGTP, dTTP (25 µM each); and 400 µM ddCTP for termination; 500 µM MgC_2 ; pH 7.5	Vial 12
Extension/termination mixture ddGTP (with 7-Deaza-dGTP, pink)	Mixture containing dATP, dCTP, 7-Deaza-dGTP, dTTP (25 µM each); and ddGTP for termination; 75 µM ddGTP; 175 µM $MgCl_2$; pH 7.5	Vial 13
Extension/termination mixture ddTTP (with 7-Deaza-dGTP, pink)	Mixture containing dATP, dCTP, 7-Deaza-dGTP, 1275 µM ddTTP (25 µM each), and ddTTP for termination; 1370 µM $MgCl_2$; pH 7.5	Vial 14
Formamide buffer solution	Stop buffer	Vial 15

Denaturation buffer	2 M NaOH, 2 mM EDTA, pH 8; sterile
Neutralization buffer	2 M ammonium acetate in water; pH 4.5, sterile. Dissolve 15.4 g ammonium acetate in approximately 50 ml redistilled water, adjust the pH to 4.5 with acetic acid (100% analytical grade), and make up to 100 ml with redist. water.
Ethanol	95% at –20°C, 70% at +4°C

Nylon membranes, positively charged	Nylon Membrane function tested with the Genius System. To cut the membrane to the size of the sequencing gel, we recommend the use of the 0.3 x 3 m membrane roll (Cat. No. 1417 240).
Genius 3 Kit or Genius 7 Kit	Colorimetric detection is performed with the substrates X-Phosphate/NBT as described on page 59. Chemiluminescent detection is performed with the substrate Lumigen PPD, Lumi-Phos 530, or Lumi-Phos Plus as described on page 56 in the "Detection" section. Both methods are performed with slight modifications.

Experimental details for the single-stranded and double-stranded sequencing reactions, gel electrophoresis and blotting, and the detection reaction itself are described here. The kit includes double-stranded template DNA (pUC18 DNA) and single-stranded template DNA (M13mp18 DNA) for 5 control reactions each. Solutions should be mixed and subjected to a short centrifugation step immediately before use.

Single-stranded DNA Sequencing

Primer annealing

1. Combine and mix the following reagents in a sterile microcentrifuge tube:
   1 µg of single-stranded template DNA (2.5 µl from vial 2) or 0.5 pmol test DNA
   1 pmol primer (1 µl from vial 3)
   2 µl reaction buffer (vial 5)
   Adjust final volume to 10 µl with redist. H_2O.
2. Centrifuge briefly.
3. Incubate at +55°C for 10 min.
4. Allow the mixture to cool slowly at room temperature, and centrifuge again briefly. While the primer annealing mixture is cooling, prepare the four extension/termination mixtures in four appropriately marked or colored reaction vials or a microtiter plate. Dispense the extension/termination mixtures separately into the 4 vials or 4 wells of the microtiter plate (2 µl each of vials 7, 8, 9, and 10; or 11, 12,13, and 14).
5. Add redist. H_2O to the cooled primer annealing mixture for a final volume of 19 µl.
6. Add 1 µl *Taq* DNA Polymerase (3 U/µl, vial 6) to the primer annealing mixture (final volume, 20 µl).

Extension/termination reaction

1. At room temperature, add 4 µl of the primer annealing mixture, from above, to each of the marked extension/termination mixtures (A, C, G, T) and, in case of vials, centrifuge briefly.
2. Incubate for 3 min at +70°C.
3. Subject the four tubes to a brief centrifugation.
4. Add 2 µl of the formamide buffer (vial 15, stop buffer), to each of the four tubes to stop the reaction.
5. These mixtures may then be stored at –20°C, or be immediately subjected to electrophoresis on a sequencing gel.

Double-stranded DNA Sequencing

Denaturation of plasmid DNA

1. Dilute 1 pmol plasmid template DNA (8 µl of control double-stranded DNA containing 2 µg pUC18 DNA, vial 1; calculate for equivalent amount of test double-stranded DNA) with redist. H_2O to a final volume of 18 µl.
2. Add 2 µl of denaturation buffer (NaOH/EDTA), mix thoroughly, and incubate for 5 min at room temperature.
3. Neutralize the mixture with 2 µl of the neutralization buffer (sodium acetate solution), and immediately continue with the next step.
4. Add 100 µl pre-chilled 95% ethanol (–20°C), mix, and transfer the reaction to –70°C for at least 5 min.
5. Centrifuge to sediment the denatured precipitated DNA at +4°C in a microcentrifuge at 10,000 x g for 15 min. Carefully decant the supernatant to avoid loosing the DNA pellet.
6. Wash the DNA pellet by adding 1 ml 70% ethanol (–20°C), briefly vortex, and centrifuge as above for 15 min. Again, carefully discard the supernatant.
7. Briefly dry the denatured plasmid DNA under vacuum.

   The DNA may be stored for a few days at –20°C.

Primer annealing

1. Add 1 pmol of either the M13/pUC sequencing primer (1 µl of vial 3) or the M13/pUC reverse sequencing primer (1 µl of vial 4) to the dry denatured plasmid DNA (from step 7 of the Denaturation of plasmid DNA procedure above). Also add 2 µl of the reaction buffer (vial no. 5), and make up the mixture to a final volume of 10 µl with redist. H_2O.
2. The procedure is completed by following all the steps as described above for single-stranded DNA sequencing, starting from step 2 of primer annealing to step 5 of the extension/termination reaction.

Electrophoresis and Blotting

The products obtained from the above reactions are subsequently denatured with formamide and heat, separated by electrophoresis on a denaturing polyacrylamide gel, and transferred to a positively charged nylon membrane. The Nylon Membrane supplied by Boehringer Mannheim has been tested for this application and is highly recommended. Other nylon membranes (*e.g.*, Biodyne A [Pall]) are also suitable but require longer exposure to X-ray film for chemiluminescence detection.

Contact blot method

This is an efficient and convenient method for the transfer of the sequence ladder from the polyacrylamide sequencing gel to a nylon membrane (see Figures 19–29).

1. Produce a polyacrylamide sequencing gel in a mold where one glass plate has been treated with Bind-Silane and the other with Repel-Silane. The gel should be of 0.2 mm thickness. A running buffer consisting of 90 mM Tris, 90 mM boric acid, and 2.5 mM EDTA, at pH 8.2 is also required. The use of a 5-fold-concentrated running buffer in the bottom chamber is recommended to reduce the mobility of the smaller DNA fragments in the lower part of the gel. The use of a comb that allows the application of at least a 3 µl volume is necessary (*e.g.*, shark tooth comb).
2. Immediately before gel electrophoresis, denature the sequencing reaction products (from step 5 of the extension/termination reaction) by heating at +95°C for 3 min. Place on ice to cool, and centrifuge briefly. At least 3 µl of each of the four elongation/termination reactions (A, C, G, T) should be separately dispensed into the appropriate slots of the sequencing gel.
3. Following electrophoresis, carefully remove one of the glass plates.
4. Cut a nylon membrane to match the size of the gel.
5. Apply the nylon membrane to the sequencing gel. Ensure good contact between the gel and the membrane, and eliminate any bubbles.
6. Cover the nylon membrane with a sheet of Whatman paper, and place a glass plate on top to complete the sandwich.
7. Load the sandwich with a weight of approximately 2 kg.
8. The transfer should be complete within 20 min.
9. Disassemble the sandwich, pull off the nylon membrane, and expose it to UV-light (3 min on a transilluminator) to fix the DNA to the membrane.

In case the membrane size is larger than the transilluminator hood, fold the membrane back without breaking it, and expose both halves of the upper (gel exposed) membrane side to UV light subsequently.

Illustration of the contact blot procedure for transfer of the sequence ladder after sequencing with DIG

Figure 19. Following sequencing gel electrophoresis, carefully remove the Repel-Silan-treated glass plate.

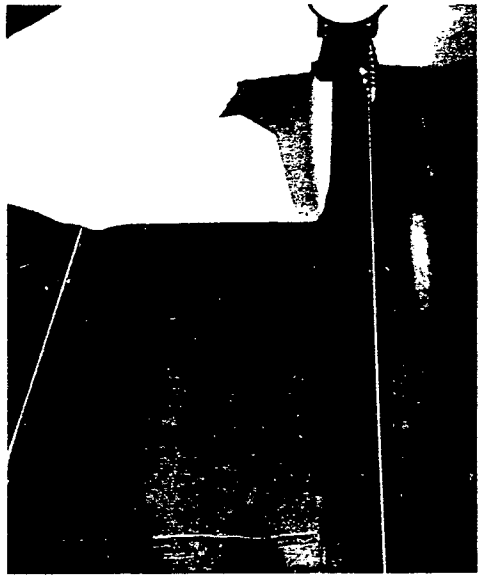

Figure 20. Apply a nylon membrane that was cut to match the size of the gel to the sequencing gel. Ensure good contact between the gel and the membrane, avoiding air bubbles.

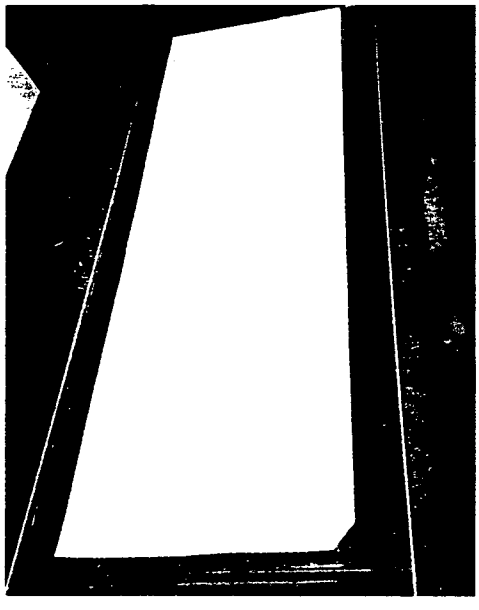

Figure 21. Cover the membrane with a sheet of Whatman 3 MM paper.

Figure 22. Cover the gel/membrane Whatman sandwich with a glass plate.

Figure 23. Load the sandwich with a weight of approximately 2 kg, evenly distributed.

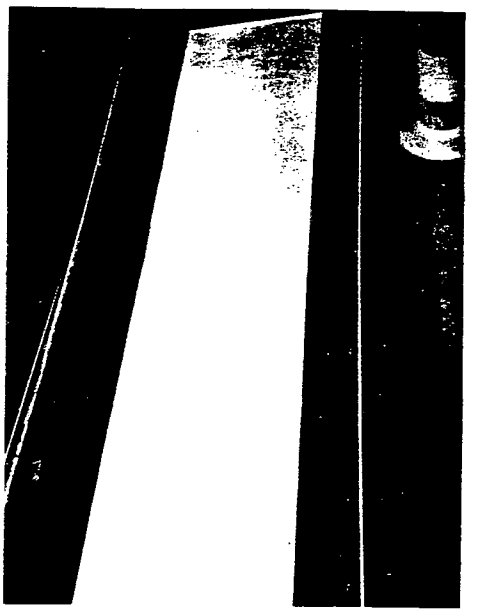

Figure 24. The transfer is complete within 20 min.

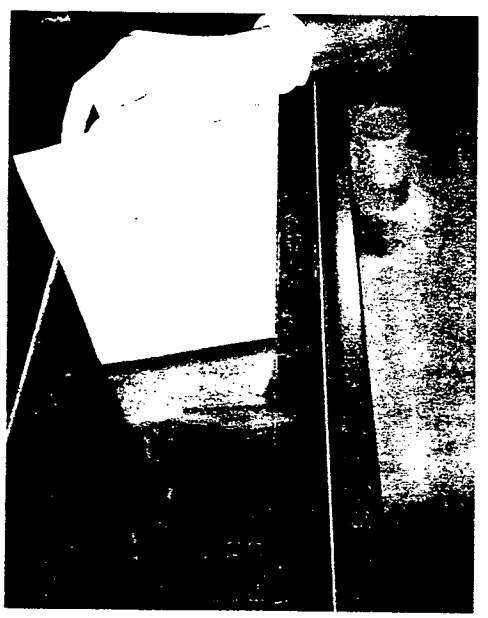

Figure 25. Pull off the Nylon Membrane from the gel.

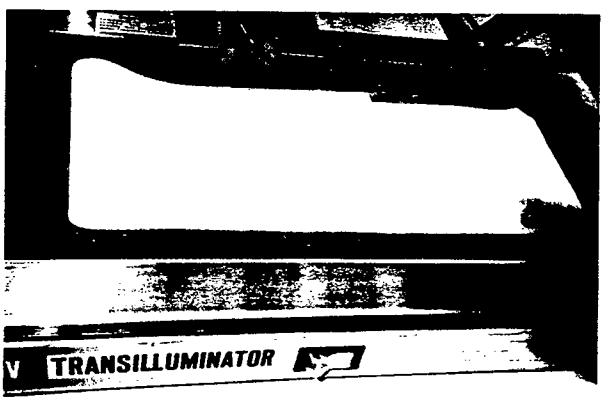

Figure 26. Expose the membrane to UV light for 3 min on a transilluminator in order to bind the DNA. In case the membrane size is larger than the transilluminator used, fold the membrane back without breaking, and expose both halves of the upper (gel exposed) membrane side to UV light subsequently.

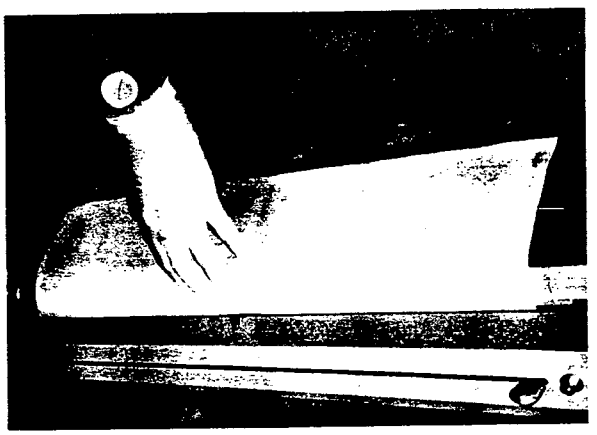

Figure 27. Seal the membrane in a plastic bag.

Figure 28. Perform all detection steps of the DIG luminescent or colorimetric detection in the plastic bag to reduce buffer volumes to approximately 10 ml/100 cm^2 membrane size. The plastic bag need not be changed during the steps of the detection procedure; it can be cut open and resealed repeatedly.

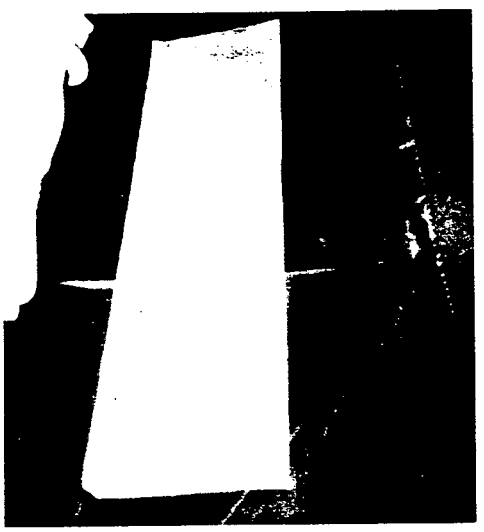

Figure 29. After performing the luminescent detection steps and subsequently drying the membrane on a Whatman 3 MM paper for a few minutes (not to complete dryness), place the nylon membrane between two nonsealed plastic foils for exposure to X-ray film.

Vacuum blot method

In this case, both glass plates of the gel mold should be treated with Repel-Silane. Use a shark tooth comb that allows the application of at least 3 µl of each reaction, as gel wells produced by standard combs tend to slip down when both plates are treated with Repel-Silane. Following electrophoresis, carefully remove one of the glass plates, and apply a nylon membrane, as described above, onto the gel. The sequencing gel, with attached nylon membrane, is then lifted off the second glass plate and transferred to a conventional gel dryer. The porous gel support surface of the dryer is covered with a layer of Whatman filter paper, then the sequencing gel/nylon membrane, with the gel above the membrane. With the heating switched off, a vacuum is then applied. Transfer should be complete in 15 min. After blotting, the nylon membrane usually remains attached to the gel. Do not attempt to separate them before performing the wash step for DIG detection. First, expose the membrane to UV light as above (the attached gel has no influence on the fixation of the DNA to the membrane).

Direct blotting electrophoresis (DBE)

The most convenient method that avoids extensive handling is through the use of a DBE (direct blotting electrophoresis) device (GATC, Konstanz, Germany or MWG-Biotech, Ebersberg, Germany). In this method, the sequencing products migrate through and out of a relatively short sequencing gel. The apparatus moves a nylon membrane at constant speed along the bottom of the gel, capturing the DNA fragments as they sequentially elute out of the gel. When working with the DBE device, the appropriate nylon membrane supplied by MWG is recommended. With this method, excellent resolution of the bands is achieved since all the fragments are subject to electrophoresis through the same length of gel. The spacing between adjacent bands is constant over a wide range.

Detection reaction

The immobilized DNA fragments can be detected via the linked digoxigenin at the 5'-end of the sequencing primer. Detection is performed using the alkaline phosphatase color substrate NBT/X-Phosphate or the chemiluminescence substrates Lumigen PPD, Lumi-Phos 530, or Lumi-Phos Plus as described in the "Detection" section of this Guide. The following modifications of the detection protocols are recommended.

All the steps should be performed in a sealed bag to reduce buffer volumes (10 ml/100 cm^2 membrane size). The plastic bag need not be changed during the different steps of the detection procedure; it can be cut open and resealed repeatedly (exception: step 10 of the chemiluminescence detection protocol, where a new bag should be used for membrane exposure). If performing staining in an open tray, use enough buffer solution to completely immerse the membrane.

In case of vacuum blot transfer, the washing step (step 1 in either detection protocol) must be performed in an open tray. The nylon membrane will detach from the gel during this process, and the gel can be removed and discarded.

All incubations should be performed with careful shaking or mixing to ensure a uniform immersion of the nylon membrane in the small buffer volumes. The use of a rocking shaker is recommended. Alternatively, a cylindrical roller of appropriate size may be used to perform all the detection reactions. Avoid membrane overlap during the incubations.

For chemiluminescence detection, as the format of blotting membranes is relatively large, we recommend the use of Lumigen PPD at a concentration of 10 µg/ml (23.5 µM, a 1:1000 dilution of Lumigen PPD stock solution), in order to reduce the cost of the detection. The use of a 1000-fold Lumigen PPD stock solution is a modification of step 10 of the procedure described in the "Detection" section recommended for hybridization experiments. An extended exposure time of 90–180 min is required to compensate for the use of the higher dilution. Do not reuse the diluted Lumigen PPD or Lumi-Phos Plus. After incubation in the Lumigen PPD substrate solution, the nylon membrane is removed from the plastic bag and dried on a Whatman 3 MM paper for a few minutes. Before complete drying, the nylon membrane is placed between two plastic foils (not sealed) and incubated for 15 min at +37°C. The sandwich is subsequently exposed to X-ray film for 90–180 min.

Colorimetric detection using X-Phosphate/NBT is usually complete after 8–16 h of incubation with the color substrates.

Quality control

The standard assay (using the control DNA gel electrophoresis and vacuum blotting) typically yields a sequence of 250 nucleotides when a single- or double-stranded template is used. Standard detection times are used as stated in the respective protocols.

Trouble Shooting for the Genius Nonradioactive DNA Sequencing Kit

DIG primers

Check the digoxigenylation of custom-made DIG sequencing primers by comparing them to the DIG sequencing primers provided by Boehringer Mannheim.

10 fmols of 5'-DIG-labeled primer should be detectable in a spot assay after chemiluminescenct detection with 5 min exposure time (using the standard chemiluminescence detection protocol).

Gel electrophoresis

As each primer extension product is labeled with only one DIG molecule, it is necessary to load at least 3 µl of each extension/termination reaction onto the sequencing gel. This results in sufficient signal intensity after chemiluminescent detection times of 90–180 min.

For this reason, always use gel combs that allow the application of at least 3 µl. For the contact blot procedure, standard combs, as well as shark tooth combs, can be used, as one of the glass plates is treated with Bind-Silane and the other with Repel-Silane before pouring the gel. For the vacuum blot method, always use a shark tooth comb as the gel wells produced by standard combs tend to slip down when both glass plates are treated with Repel-Silane.

Membrane

We recommend the use of the Boehringer Mannheim Nylon Membrane (roll of 0.3 x 3 m, Cat. No. 1417 240) when performing the contact blot or vacuum blot procedure from standard sequencing gels. The Boehringer Mannheim Nylon Membrane shows enhanced signal intensity compared to other nylon membranes after exposure of sequence ladders from the above-mentioned blotting procedures.

Cross-linking of transferred sequence ladder

As the format of the membrane might be larger than the format of commercially available crosslinkers, a standard transilluminator (for viewing Ethidium bromide-stained gels) can be used as well. The fixation time is not critical, as no hybridization step is performed subsequently. Fix for at least 3 min. In case the membrane size is larger than the transilluminator used, fold the membrane back without breaking it, and expose both halves of the upper, gel-exposed membrane side to UV-light subsequently.

Do not try to fix the sequence ladder by baking the membrane in an oven for 30 min at +120°C (as recommended in the standard DIG detection protocol for unlabeled DNA before hybridization). For labeled sequencing products, this procedure leads to a drastic decrease in detection sensitivity.

Blotting/detection

With the vacuum blot procedure, the gel usually sticks to the nylon membrane. Do not try to remove it before performing the washing step for DIG detection. Do not forget to add 0.3% Tween® 20 to the washing buffer. This enables the gel to be detached from the membrane and to be removed from the washing buffer.

Do not let the membrane(s) overlap during the detection steps as this causes limited access of the detection reagents to the surface of the membrane and thus lower sensitivity of the signals obtained.

Chemiluminescent detection

Re-exposure of the sequence ladder to X-ray film several hours after performing the chemiluminescent detection steps (*e.g.*, the next morning) shortens the required exposure time by a factor of 2–3, compared to the exposure time required immediately after the preincubation step, due to the kinetics of light emission of the chemiluminescent substrate.

Appendix A *Genius Kits' Contents*

Genius 1 DNA Labeling and Detection Kit **1093 657**

1	one vial containing 20 µl **Unlabeled Control DNA 1** (100 µg/ml mixture of pBR328 DNA digested separately with *Eco* RI, *Bgl* I, and *Hinf* I. The separate digests are combined in a ratio of 2:3:3. Sizes [in basepairs] of the 16 pBR328 fragments are 4907, 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 298, 234, 234, 220, 154, 154) in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0	used as a control target in a Southern blot	
2	one vial containing 20 µl **Unlabeled Control DNA 2** (200 µg/ml pBR328 DNA that has been linearized with *Eco* RI)	used to practice labeling and to check labeling efficiency	
3	one vial containing 1 ml **DNA dilution buffer** (50 µg/ml herring sperm DNA, in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 [+25°C])	used to prepare dilutions of the Labeled Control DNA (or experimental DNA)	
4	one vial containing 50 µl **Labeled Control DNA** (digoxigenin-labeled pBR328 DNA that has been random prime labeled according to the standard labeling procedure; the total DNA concentration in the vial is 20 µg/ml, but only 5 µg/ml of it is DIG-labeled DNA.)	used to estimate the yield of DIG-labeled DNA	1585 738
5	one vial containing 50 µl **Hexanucleotide mixture (10X)** (62.5 A_{260} units/ml random hexanucleotides, 500 mM Tris-HCl, 100 mM $MgCl_2$, 1 mM Dithioerythritol [DTE], 2 mg/ml BSA; pH 7.2)	contains hexamers and reaction buffer for the labeling reaction	1277 081
6	one vial containing 50 µl **dNTP labeling mixture (10X)** (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM alkali-labile DIG-dUTP, pH 6.5)	component of the labeling reaction	1277 065
7	one vial containing 25 µl **Klenow enzyme, labeling grade** (2 units/µl DNA Polymerase I [Klenow enzyme, large fragment])	synthesizes DIG-labeled DNA	1008 404
8	one vial containing 200 µl **Anti-DIG-alkaline phosphatase (<DIG> AP-Conjugate)** (750 units/ml polyclonal sheep anti-digoxigenin Fab fragments, conjugated to alkaline phosphatase)	binds to incorporated digoxigenin	1093 274
9	two vials, each containing 1.25 ml **NBT** (75 mg/ml nitroblue tetrazolium salt in 70% [v/v] dimethylformamide)	precipitating substrate used to locate alkaline phosphatase-conjugated anti-DIG	1383 213 (100 mg/ml; dilute prior to use)
10	two vials, each containing 0.9 ml **X-phosphate solution** (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt in 100% dimethylformamide)	precipitating substrate used to locate alkaline phosphatase-conjugated anti-DIG	1383 221
11	two bottles, each containing 50 g **Blocking reagent** (Blocking reagent for nucleic acid hybridization; white powder)	blocks nonspecific binding of probes	1096 176 (50 g)

Genius 2 DNA Labeling Kit

1175 033

1	one vial containing 20 µl **Unlabeled Control DNA 1** (100 µg/ml mixture of pBR328 DNA digested separately with *Eco* RI, *Bgl* I, and *Hinf* I. The separate digests are combined in a ratio of 2:3:3. Sizes [in basepairs] of the 16 pBR328 fragments are 4907, 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 298, 234, 234, 220, 154, 154) in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0	used as a control target in a Southern blot	
2	one vial containing 20 µl **Unlabeled Control DNA 2** (200 µg/ml pBR328 DNA that has been linearized with *Eco* RI)	used to practice labeling and to check labeling efficiency	
3	one vial containing 1 ml **DNA dilution buffer** (50 µg/ml herring sperm DNA, in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 [+25°C])	used to prepare dilutions of the Labeled Control DNA (or experimental DNA)	
4	one vial containing 50 µl **Labeled Control DNA** (digoxigenin-labeled pBR328 DNA that has been random prime labeled according to the standard labeling procedure; the total DNA concentration in the vial is 20 µg/ml, but only 5 µg/ml of it is DIG-labeled DNA.)	used to estimate the yield of DIG-labeled DNA	1585 738
5	one vial containing 80 µl **Hexanucleotide mixture (10X)** (62.5 A_{260} units/ml random hexanucleotides, 500 mM Tris-HCl, 100 mM $MgCl_2$, 1 mM Dithioerythritol [DTE], 2 mg/ml BSA; pH 7.2)	contains hexamers and reaction buffer for the labeling reaction	1277 081
6	one vial containing 80 µl **dNTP labeling mixture (10X)** (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM alkali-labile DIG-dUTP, pH 6.5)	component of the labeling reaction	1277 065
7	one vial containing 40 µl **Klenow enzyme, labeling grade** (2 units/µl DNA Polymerase I [Klenow enzyme, large fragment])	synthesizes DIG-labeled DNA	1008 404

Genius 3 Nucleic Acid Detection Kit

1175 041

1	one vial containing 50 µl **Labeled Control DNA** (digoxigenin-labeled pBR328 DNA that has been random prime labeled according to the standard labeling procedure; the total DNA concentration in the vial is 20 µg/ml, but only 5 µg/ml of it is DIG-labeled DNA.)	used to estimate the yield of DIG-labeled DNA and used to practice detecting DIG-labeled DNA	1585 738
2	one vial containing 1 ml **DNA dilution buffer** (50 µg/ml herring sperm DNA, in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 [+25°C])	used to prepare dilutions of the Labeled Control DNA (or experimental DNA)	
3	one vial containing 200 µl **Anti-DIG-alkaline phosphatase (<DIG> AP-Conjugate)** (750 units/ml polyclonal sheep anti-digoxigenin Fab fragments, conjugated to alkaline phosphatase)	binds to incorporated digoxigenin	1093 274
4	two vials, each containing 1 ml **NBT** (75 mg/ml nitroblue tetrazolium salt in 70% [v/v] dimethylformamide)	precipitating substrate used to locate alkaline phosphatase-conjugated anti-DIG	1383 213 (100 mg/ml; dilute prior to use)
5	two vials, each containing 0.75 ml **X-phosphate solution** (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt in 100% dimethylformamide)	precipitating substrate used to locate alkaline phosphatase-conjugated anti-DIG	1383 221
6	two bottles, each containing 50 g **Blocking reagent** (Blocking reagent for nucleic acid hybridization; white powder)	blocks nonspecific binding of probes	1096 176 (50 g)

1	one vial containing 40 µl **pSPT18 DNA** (0.25 mg/ml)	cloning/transcription vector; subclones are transcribed into RNA probes by T7 or SP6 RNA polymerase	909 793
2	one vial containing 40 µl **pSPT19 DNA** (0.25 mg/ml)	cloning/transcription vector; subclones are transcribed into RNA probes by T7 or SP6 RNA polymerase	909 815
3	one vial containing 20 µl **Control DNA 1, pSPT18-Neo** (0.25 mg/ml pSPT18-Neo DNA, cleaved with *Pvu* II)	used to practice making RNA probes with T7 RNA polymerase; results in DIG-labeled "antisense" Neo transcripts 760 bases in length	
4	one vial containing 20 µl **Control DNA 2, pSPT19-Neo** (0.25 mg/ml pSPT19-Neo DNA, cleaved with *Pvu* II)	used to practice making RNA probes with SP6 RNA polymerase; results in DIG-labeled "antisense" Neo transcripts 760 bases in length	
5	one vial containing 100 µl **Labeled Control RNA** (10 µg of digoxigenin-labeled "antisense" Neo RNA made with T7 RNA polymerase from 1 µg of Control DNA 1. Reaction products were phenol extracted, ethanol precipitated, and resuspended in 100 µl of DEPC-treated water. Template DNA is still present in the vial.)	used to estimate the yield of DIG-labeled RNA and used for hybridization with Unlabeled Control RNA (vial 6)	1585 746
6	one vial containing 20 µl **Unlabeled Control RNA** (200 µg/ml unlabeled Neo poly(A) "sense" RNA, in DEPC-treated H_2O. The Neo poly(A) RNA is approximately 1 kb in length.)	target RNA used to practice RNA/RNA hybridizations; when applied to a membrane, this RNA will hybridize with the Labeled Control RNA (Vial 5)	
7	one vial containing 40 µl **NTP labeling mixture (10X)** (10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP 3.5 mM DIG-UTP; in Tris-HCl, pH 7.5 [+20°C])	component of the labeling reaction	1277 073
8	one vial containing 40 µl **10X transcription buffer** (400 mM Tris-HCl, pH 8.0; 60 mM $MgCl_2$; 100 mM dithioerythritol [DTE]; 20 mM spermidine) 100 mM NaCl, 1 unit/ml RNase inhibitor)	component of the labeling reaction	
9	one vial containing 20 µl **DNase I, RNase-free** (10 units/µl)	degrades DNA template after the labeling reaction	776 785
10	one vial containing 20 µl **RNase inhibitor** (20 units/µl)	prevents the degradation of RNA during the labeling reaction	799 017
11	one vial containing 20 µl **SP6 RNA Polymerase** (20 units/µl)	synthesizes RNA from a DNA template	810 266
12	one vial containing 20 µl **T7 RNA Polymerase** (20 units/µl)	synthesizes RNA from a DNA template	881 767

Genius 5 Oligonucleotide 3'-End Labeling Kit **1362 372**

Vial	Description	Function	Cat. No.
1	one vial containing 100 µl **5X reaction buffer** (1 M potassium cacodylate*, 125 mM Tris-HCl, 1.25 mg/ml bovine serum albumin; pH 6.6 +25°C])	optimized buffer for terminal transferase	1243 276
2	one vial containing 100 µl **$CoCl_2$ solution** (25 mM cobalt chloride)	cofactor required for optimal terminal transferase activity	1243 306
3	one vial containing 25 µl **DIG-ddUTP** (1 mM digoxigenin-11-ddUTP [2',3' dideoxy-uridine-5'-triphosphate coupled to digoxigenin via an 11-atom spacer arm]) in redistilled water	digoxigenin-labeled nucleotide used for the addition of a single residue on to the 3' end of an oligonucleotide	1363 905
4	one vial containing 25 µl **terminal transferase** (50 units/µl, in 200 mM potassium cacodylate*, 1 mM EDTA, 200 mM KCl, 0.2 mg/ml bovine serum albumin; 50% [v/v] glycerol; pH 6.5 [+25°C])	catalyzes addition of nucleotides to the 3' end of an oligonucleotide	220 582 (sold separately at 25 U/µl)
5	one vial containing 25 µl **Control Oligonucleotide, Unlabeled** (30-mer, 5'-p TTG GGT AAC GCC AGG GTT TTC CCA GTC ACG OH-3', homologous to the *lac* Z' region in pUC and M13 plasmids), 20 pmol/ml, in redistilled water	used to practice labeling and to check labeling efficiency	
6	one vial containing 100 µl **Control Oligonucleotide, DIG-ddUTP-labeled** (2.5 pmol/µl; [sequence as in vial 5] labeled with DIG-ddUTP under standard kit assay reaction conditions) in redistilled water	used to estimate the yield of DIG-labeled oligonucleotide and used as a probe for Control DNA (Vial 7)	1585 754
7	one vial containing 20 µl **Control DNA** (0.25 mg/ml pUC18 DNA [supercoiled], in 10 mM Tris-HCl, 1 mM EDTA; pH 7.6 [+25°C])	used as a hybridization target for the Control Oligonucleotides (Vial 5 or 6)	885 797
8	one vial containing 50 µl **glycogen solution** (20 mg/ml glycogen solution) in redistilled water	used as a carrier to increase the recovery of oligonucleotide after the labeling reaction	901 393
9	one vial containing 1 ml **DNA dilution buffer** (50 µg/ml herring sperm DNA, in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 [+25°C]	used to prepare dilutions of the DIG-ddUTP-labeled Control Oligonucleotide (or experimental oligonucleotide)	

*Potassium cacodylate is toxic. Wear gloves when handling. Discard as regulated for toxic waste.

Genius 6 Oligonucleotide Tailing Kit **1417 231**

1	one vial contaiing 100 µl **5X reaction buffer** (1 M potassium cacodylate*, 125 mM Tris-HCl, 1.25 mg/ml bovine serum albumin; pH 6.6 [+25°C])	optimized buffer for terminal transferase	1243 276
2	one vial containing 100 µl **$CoCl_2$ solution** (25 mM cobalt chloride)	cofactor required for optimal terminal transferase activity	1243 306
3	one vial containing 25 µl **DIG-dUTP solution** (1 mM digoxigenin-11-dUTP [2'-deoxy-uridine-5'-triphosphate, coupled to digoxigenin via an 11-atom spacer arm], alkali stable) in redistilled water	digoxigenin-labeled nucleotide used for the addition of multiple residues to the 3' end of an oligonucleotide	1093 088
4	one vial containing 25 µl **dATP solution** (10 mM dATP solution; in Tris buffer, pH 7.5; solution of the crystallized disodium salt)	unlabeled nucleotide used to facilitate the addition of multiple DIG-dUTP residues to the 3' end of an oligonucleotide	103 977
5	one vial containing 25 µl **terminal transferase** (50 units/µl, in 200 mM potassium cacodylate*, 1 mM EDTA, 200 mM KCl, 0.2 mg/ml bovine serum albumin; 50% [v/v] glycerol; pH 6.5 [+25°C])	enzyme used to catalyze addition of nucleotides to the 3' end of an oligo	220 582 (sold separately at 25 U/µl)
6	one vial containing 25 µl **Control Oligonucleotide, Unlabeled** (30-mer, 5'-p TTG GGT AAC GCC AGG GTT TTC CCA GTC ACG OH-3', homologous to the *lac* Z' region in pUC and M13 plasmids), 20 pmol/ml, in redistilled water	used to practice labeling and used to check labeling efficiency	
7	one vial containing 100 µl **Control Oligonucleotide, DIG-dUTP/dATP-tailed** (2.5 pmol/µl; [sequence as in vial 6] tailed with DIG-dUTP/dATP under standard kit assay reaction conditions) in redistilled water	used to estimate the yield of DIG-labeled oligonucleotide	
8	one vial containing 20 µl **Control DNA** (0.25 mg/ml pUC18 DNA [supercoiled], in 10 mM Tris-HCl, 1 mM EDTA; pH 7.6 [+25°C])	used as a hybridization target for the Control Oligonucleotides (vial 6 or 7)	885 797
9	one vial containing 50 µl **glycogen solution** (20 mg/ml glycogen solution) in redistilled water	used as a carrier to increase the recovery of oligonucleotide after the labeling reaction	901 393
10	one vial containing 1 ml **DNA dilution buffer** (50 µg/ml herring sperm DNA, in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 [+25°C])	used to prepare dilutions of the DIG-dUTP/dATP-tailed Control Oligonucleotide (or experimental oligonucleotide)	
11	one vial containing 1 ml **poly(A)** (10 mg/ml in H_2O) This amount of poly(A) is sufficient for the preparation of 100 ml hybridization solution and should be used for hybridization with the Control Oligonucleotide as well, in which case poly(dA) is not necessary.	prevents nonspecific hybridization	108 626

*Potassium cacodylate is toxic. Wear gloves when handling. Discard as regulated for toxic waste.

Genius 7 Luminescent Detection Kit

1363 514

Vial	Description	Function	Cat. No.
1	one vial containing 50 µl **Labeled Control DNA** (digoxigenin-labeled pBR328 DNA that has been random prime labeled according to the standard labeling procedure; the total DNA concentration in the vial is 20 µg/ml, but only 5 µg/ml of it is DIG-labeled DNA.)	used to estimate the yield of DIG-labeled DNA and used to practice detecting DIG-labeled DNA	1585 738
2	one vial containing 1 ml **DNA dilution buffer** (50 µg/ml herring sperm DNA, in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 [+25°C])	used to prepare dilutions of the Labeled Control DNA (or experimental DNA)	
3	one vial containing 100 µl **Anti-DIG-alkaline phosphatase (<DIG>AP-Conjugate)** (750 units/ml polyclonal sheep anti-digoxigenin Fab fragments, conjugated to alkaline phosphatase)	binds to incorporated digoxigenin	1093 274
4	two bottles, each containing 50 g **Blocking reagent** (Blocking reagent for nucleic acid hybridization; white powder)	blocks nonspecific binding of probes	1096 176 (50 g)
5	one vial containing 1 ml **Lumigen PPD** (10 mg/ml; 23.5 mM [4-methoxy-4-(3-phosphatephenyl)-spiro-(1,2-dioxetane-3,2'-adamantane) disodium salt])	chemiluminescent substrate used to locate alkaline phosphatase-conjugated anti-DIG	1357 328

Genius 8 Oligonucleotide 5'-End Labeling Set

1480 863

Vial	Description	Function	Cat. No.
1	one vial containing 100 µl **[N-Trifluoroacetamido-(3-oxa)-pentyl-N,N-diisopropyl-methyl]-phosphoramidite** (aminolinker)*	reacts with oligonucleotide in the final synthesis step according to the solid-phase phosphoramidite method	
2	five vials, each containing 1 mg **Digoxigenin-3-O-methylcarbonyl-ε aminocaproic acid-N-hydroxy-succinimide ester** (DIG-NHS-ester)*†	used to introduce DIG moiety to the 5' end	1333 054

* The aminolinker reacts violently with water, and it is irritating to eyes, respiratory system, and skin.

† DIG-NHS ester is very toxic by inhalation, in contact with skin, or swallowed. Do not breath dust.

1	one vial containing 40 µl **Control DNA, double-stranded** template plasmid DNA, pUC18 DNA with 40 µl pUC18 DNA, 0.25 µg/µl in Tris-EDTA buffer (TE buffer); pH 8	template for control sequencing reaction
2	one vial containing 25 µl **Control DNA, single-stranded template DNA,** M13mp18 DNA with 25 µl M13mp18 ssDNA, 0.2 µg/µl in TE buffer pH 8	template for control sequencing reaction
3	one vial containing 110 µl **M13/pUC sequencing primer**, 17-mer sequencing primer with the 5'-end labeled with digoxigenin 110 µl M13/pUC digoxigenin-labeled, 1 pmol/µl in water.	primer for sequencing reaction
4	one vial containing 110 µl **M13/pUC reverse sequencing primer** 17-mer with the 5'-end labeled with digoxigenin reverse sequencing primer with 110 µl M13/pUC digoxigenin-labeled, 1 pmol/µl in water.	primer for sequencing reaction
5	one vial containing 250 µl **Reaction buffer**	buffer for the hybridization and chain elongation reaction
6	one vial containing 110 µl ***Taq* DNA Polymerase** DNA polymerase for the chain elongation reaction with 110 µl *Taq* DNA Polymerase, 3 units/µl.	component of the sequencing reaction
7	one vial containing 220 µl **Extension/termination mixture ddATP** (with dGTP, green), mixture containing dATP, dCTP, dGTP, dTTP, and ddATP for termination reaction.	nucleotide mix for standard templates
8	one vial containing 220 µl **Extension/termination mixture ddCTP** (with dGTP, green), mixture containing dATP, dCTP, dGTP, dTTP, and ddCTP for termination reaction.	nucleotide mix for standard templates
9	one vial containing 220 µl **Extension/termination mixture ddGTP** (with dGTP, green), mixture containing dATP, dCTP, dGTP, dTTP, and ddGTP for termination reaction.	nucleotide mix for standard templates
10	one vial containing 220 µl **Extension/termination mixture ddTTP** (with dGTP, green), mixture containing dATP, dCTP, dGTP, dTTP, and ddTTP for termination reaction.	nucleotide mix for standard templates
11	one vial containing 220 µl **Extension/termination mixture ddATP** (with 7-Deaza-dGTP, pink), mixture containing dATP, dCTP, 7-Deaza-dGTP, dTTP, and ddATP for termination reaction	nucleotide mix for GC-rich templates
12	one vial containing 220 µl **Extension/termination mixture ddCTP** (with 7-Deaza-dGTP, pink), mixture containing dATP, dCTP, 7-Deaza-dGTP, dTTP, and ddCTP for termination reaction.	nucleotide mix for GC-rich templates
13	one vial containing 220 µl **Extension/termination mixture ddGTP** (with 7-Deaza-dGTP, pink), mixture containing dATP, dCTP, 7-Deaza-dGTP, dTTP, and ddGTP for termination reaction.	nucleotide mix for GC-rich templates
13	one vial containing 220 µl **Extension/termination mixture ddTTP** (with 7-Deaza-dGTP, pink), mixture containing dATP, dCTP, 7-Deaza-dGTP, dTTP, and ddTTP for termination reaction.	nucleotide mix for GC-rich templates
13	one vial containing 1 ml **Formamide buffer solution**	stops the reaction

Appendix B *Preparation of Additionally Required Solutions and Buffers*

1. DNA/Southern Blotting and Hybridization Required Solutions and Buffers

DNA dilution buffer
10 mM Tris-HCl
1 mM EDTA, pH 8.0
50 µg/ml herring sperm DNA

Depurination solution
0.25 M HCl

Denaturation solution 1
(for Southern transfer and plaque hybridization):
0.5 N NaOH 1.5 M NaCl

Denaturation solution 2
(for colony hybridization):
0.5 N NaOH 1.5 M NaCl 0.1% SDS

Neutralization solution 1
(for Southern transfer):
0.5 M Tris-HCl, pH 7.5
3 M NaCl

Neutralization solution 2
(for colony and plaque hybridization):
1.0 M Tris-HCl, pH 7.5
1.5 M NaCl

20X SSC stock solution
3 M NaCl
0.3 M sodium citrate
pH 7.0 (+20°C), autoclaved

N-lauroylsarcosine stock solution
10% (w/v) in H_2O
filtered through a 0.2–0.45 µm membrane

SDS stock solution
20% (w/v) in H_2O
filtered through a 0.2–0.45 µm membrane

Blocking reagent stock solution (5X)
Blocking Reagent is dissolved in buffer 1 to a final concentration of 10% (w/v) with shaking and heating. See Detection section for detailed instructions.

Standard hybridization buffer
5X SSC
0.1 % N-lauroylsarcosine
0.02% SDS
1% Blocking Reagent (from the 10% Blocking Reagent stock solution)

Standard hybridization buffer + 50% formamide
5X SSC
50% formamide, deionized
0.1% sodium-lauroylsarcosine
0.02% SDS
2% Blocking Reagent.

High SDS concentration hybridization buffer
7% SDS
50% formamide, deionized*
5X SSC
2% Blocking Reagent
50 mM sodium-phosphate, pH 7.0
0.1% N-lauroylsarcosine

For preparation of 500 ml of high SDS hyb buffers from stock solutions, combine the substances in the following order:

100% formamide, deionized*	250 ml
30x SSC	83 ml
1 M sodium-phosphate, pH 7.0	25 ml
10% blocking solution	100 ml
10% N-lauroylsarcosine	5 ml

Pour the solution into an Erlenmeyer flask containing 35 g SDS (attention: wear respiratory protection). Heat the solution while stirring to dissolve the SDS, then fill up to 500 ml with autoclaved H_2O. The solution can be stored at 20°C and reused after heating to +65°C.

Probe stripping solution
(for alkali-labile dUTP)
0.1–0.5 N NaOH
0.1% SDS

carbonate buffer
60 mM Na_2CO_3
40 mM $NaHCO_3$, pH 10.2

RNA dilution buffer
Mix H_2O, 20X SSC, and formaldehyde in the ratio of 5:3:2 respectively. The H_2O and 20X SSC should have been treated with diethylpyrocarbonate to destroy RNase activity.

RNA loading buffer
Make up a fresh solution.
250 µl formamide, deionized*
83 µl formaldehyde 37 % (w/v)
50 µl 10x MOPS buffer
0.01% (w/v) bromophenol blue
50 µl glycerol
Fill up to 500 µl with DEPC-treated H_2O.

10X MOPS
200 mM Morpholinopropansulfonic acid
50 mM sodium acetate
10 mM EDTA
pH 7.0. Make up in sterile H_2O or autoclave.
After autoclaving, the solution will turn yellow.

Standard hybridization buffer + formamide
Recommended for RNA probes.
50% formamide, deionized*
5X SSC
2% Blocking Reagent
0.1% (w/v) N-lauroylsarcosine
0.02% (w/v) SDS

High SDS hyb Solution
Recommended for DNA probes.

Probe stripping solution
60% formamide
50 mM Tris-HCl, pH 8.0
1% (w/v) SDS.

or

DEPC H_2O
0.1% SDS (w/v)

Transcription buffer, 10X
400 mM Tris-HCl, pH 8.0
60 mM $MgCl_2$
100 mM dithiothreitol (DTT)
20 mM spermidine
100 mM NaCl
1 unit/ml RNase inhibitor
This buffer is stable for 1 year when stored at –20°C.

Maelic acid buffer (Buffer 1)
0.1 M Maleic acid
0.15 M NaCl
pH 7.5 (+20°C)
Adjust pH with concentrated or solid NaOH; autoclave.

Washing buffer (Buffer 1 + Tween® 20)
Add 0.3% (w/v) Tween 20 to Buffer 1.
Do not autoclave Buffer 1 containing Tween 20.

Maleic acid is available from Serva (Cat. No. 28337) and Sigma (Cat. No. MD375).

Blocking Reagent stock solution
Blocking Reagent is dissolved in buffer 1 to a final concentration of 10% (w/v) with stirring and heating either on a stir plate or in a microwave oven. The Blocking Reagent must be heated while it dissolves in the maleic acid buffer. Boiling will cause the reagent to coagulate, so care should be taken to **AVOID BOILING** during this step. This will be a turbid solution.

1A. Add 10 g Blocking Reagent to 100 ml Buffer 1. Place on stir plate and heat to 60°C for approximately 1 hour or until completely in solution. If necessary, the temperature may be raised to get the last of the blocking reagent into solution.

or

1B. Dissolve 10 g Blocking Reagent in 100 ml buffer with several 30 sec heat pulses in the microwave (3 to 4 min total). **Note:** If Blocking Reagent doesn't go into solution, check pH of solution, adjust if necessary, and reapply to heat.

2. If necessary, treat with 0.1% DEPC (diethylpyrocarbonate) to destroy RNases.

3. Autoclave the solution using a regular program, such as that used for the sterilization of cell culture medium. **Note:** Blocking reagent must be completely in solution before autoclaving.

4. Store autoclaved solution at room temperature (unopened), +4°C or 20°C.

5. Check before each use for contamination.

Blocking buffer
Dilute 5X stock with Maleic acid buffer

Detection buffer (Buffer 3)
100 mM Tris-HCl, pH 9.5 (+20°C)
100 mM NaCl
50 mM $MgCl_2$

Prepare Detection buffer from sterile stock solutions to avoid $MgCl_2$ precipitation, and filter.
Note on Detection buffer: adjust the pH to 9.5 prior to addition of $MgCl_2$ otherwise $Mg(OH)_2$ will precipitate. Do not autoclave solutions of $MgCl_2$. $MgCl_2$ may be omitted if reprobing RNA blots.

TE buffer
100 mM Tris-HCl
1 mM EDTA
pH 8.0 (+20°C)

Color substrate solution (freshly prepared)
45 µl NBT solution and 35 µl X-Phosphate solution are added to 10 ml buffer 3.

Lumigen™ PPD solution
Dilute stock solution of Lumigen PPD (sold premixed at 10 mg/ml) 1:100 in buffer 3.

Lumi-Phos™ 530
Solution is ready to use.

EDTA solution
50 mM EDTA, pH 8.0

20X SSC
3 M NaCl
300 mM sodium citrate, pH 7.0

Washing solution 2X
2X SSC
0.1% SDS

Washing solution 0.5X
0.5X SSC
0.1% SDS

Washing solution 0.1X
0.1X SSC
0.1% SDS

N-lauroylsarcosine
10% (w/v) in sterile H_2O
filtered through a 0.2–0.45 µm membrane

SDS
20% (w/v) in sterile H_2O
filtered through a 0.2–0.45 µm membrane

EDTA solution
50 mM EDTA; pH 8

Formamide
Deionization of formamide
50 g ion exchange: AG 501-X8 Resin (Biorad)
500 ml formamide

Stir 30 min slowly on a stirrer, then remove resin by filtration and store the deionized formamide at 20°C.

Tris Buffers
Buffer 1
100 mM Tris
150 mM NaCl, pH 7.5.

Buffer 2
Buffer 1 with 2% w/v Blocking Reagent

Appendix C *Genius System Product Listing*

Genius™ Nonradioactive Nucleic Acid Labeling and Detection System

Kits

Genius 1, DNA Labeling and Detection	1093 657	25 labeling reactions and 50 blots (10 cm x 10 cm)
Genius 2, DNA Labeling	1175 033	40 reactions
Genius 3, Nucleic Acid Detection	1175 041	40 blots (10 cm x 10 cm)
Genius 4, RNA Labeling	1175 025	2 x 10 reactions
Genius 5, Oligonucleotide 3'-End Labeling	1362 372	25 reactions
Genius 6, Oligonucleotide Tailing	1417 231	25 reactions
Genius 7, Luminescent Detection	1363 514	50 blots (10 cm x 10 cm)
Genius 8, Oligonucleotide 5'-End Labeling Set	1480 863	1 set
PCR DIG Probe Synthesis Kit	1636 090	25 reactions

Labeling Reagents

Digoxigenin-11-dUTP, alkali-stable (1 mM solution)		1093 088 1558 706	25 nmol (25 µl) 125 nmol (125 µl)
Digoxigenin-11-dUTP, alkali-labile (1 mM solution)		1573 152 1573 179	25 nmol (25 µl) 125 nmol (125 µl)
Digoxigenin-11-UTP (10 mM solution)		1209 256	250 nmoles (25 µl)
Digoxigenin-11-ddUTP (1 mM solution)		1363 905	25 nmoles (25 µl)
Digoxigenin-16-dATP		1558 714	2.5 nmol (25 µl)
Digoxigenin DNA Labeling Mixture		1277 065	50 µl
Digoxigenin RNA Labeling Mixture		1277 073	40 µl
Digoxigenin-NHS-Ester		1333 054	5 mg
Hexanucleotide Mixture (10X)		1277 081	100 µl
High Prime Digoxigenin DNA Labeling Mix		1585 606	160 µl (40 reactions)
High Prime Biotin DNA Labeling Mix		1585 592	100 µl (25 reactions)
High Prime Fluorescein DNA Labeling Mix		1585 622	100 µl (25 reactions)
***Taq* DNA polymerase** (free 10X PCR buffer included)	5 U/µl	1146 165 1146 173 1418 432 1596 594 1435 094	100 U 500 U 4 x 250 U 2500 U Bulk
	1 Uµ/l	1647 679 1647 687	250 U 4 x 250 U

Nucleic Acid Detection and Hybridization Reagents

Anti-digoxigenin-alkaline phosphatase conjugate	1093 274	150 U
Anti-digoxigenin-β-galactosidase, Fab fragments	1634 020	150 U
Anti-digoxigenin-fluorescein conjugate	1207 741	200 µg
Anti-digoxigenin-AMCA conjugate	1533 878	200 µg
Anti-digoxigenin-peroxidase conjugate	1207 733	150 U
Anti-digoxigenin-POD (poly), Fab fragments	1633 716	50 U
Anti-digoxigenin-rhodamine conjugate	1207 750	200 µg
Anti-digoxigenin-unconjugated (Fab fragment)	1214 667	1 mg
Anti-digoxigenin-unconjugated (whole antibody, monoclonal)	1333 062	100 µg
Anti-digoxigenin-unconjugated (whole antibody, sheep polyclonal)	1333 089	200 µg
Anti-DIG-gold	1450 590	120 µg (1 ml)
Blocking Reagent for Nucleic Acid Hybridization	1096 176	50 g
DIG Easy Hyb	1603 558	500 ml
Genius Wash and Block Buffer Set	1585 762	1 set (at least 30 blots, 100 cm^2)
Lumi-Phos® 530	1413 155 1275 470 1413 163	40 ml 100 ml 200 ml
Lumi-Phos® Plus	1581 082 1581 104	50 ml 100 ml
Lumigen® PPD	1357 328	1 ml (100X conc.)
Multi-Color DNA Detection Set	1465 341	1 set (3 x 50 tablets)
Nylon membranes, positively charged	1209 299 1209 272 1417 240	20 sheets 10 x 15 cm 10 sheets 20 x 30 cm 1 roll (3 x 0.3 m)
Silver Enhancement Reagents	1465 350	30 ml developer and 30 ml enhancer

DIG-Labeled Controls

DIG-labeled control DNA	1585 738	250 ng (50 µl)
DIG-labeled control RNA	1585 746	5 µg (50 µl)
DIG-labeled control oligonucleotide	1585 754	125 pmol (50 µl)

DIG Labeled Molecular Weight Markers

Product	Cat. No.	Size
DNA Molecular Weight Marker II, digoxigenin-labeled	1218 590	5 µg
DNA Molecular Weight Marker III, digoxigenin-labeled	1218 603	5 µg
DNA Molecular Weight Marker VI, digoxigenin-labeled	1218 611	5 µg
DNA Molecular Weight Marker VIII, digoxigenin-labeled	1449 451	5 µg
DNA Molecular Weight Marker XI, digoxigenin-labeled	1465 422	2 µg (200 µl)
RNA Molecular Weight Marker I, digoxigenin-labeled	1526 529	4 µg (200 µl)
RNA Molecular Weight Marker II, digoxigenin-labeled	1526 537	2 µg (200 µl)
RNA Molecular Weight Marker III, digoxigenin-labeled	1373 099	2 µg (200 µl)

Nonradioactive Sequencing

Product	Cat. No.	Size
Genius Nonradioactive DNA Sequencing Kit	1449 443	1 kit (100 reactions)
SP6 promoter-specific primer, 5' DIG-labeled	1573 195	100 pmol
T3 promoter-specific primer, 5' DIG-labeled	1573 195	100 pmol
T7 promoter-specific primer, 5' DIG-labeled	1573 217	100 pmol
λgt11 sequencing primer, 5' DIG-labeled	1573 225	100 pmol
λgt11 reverse sequencing primer, 5' DIG-labeled	1573 233	100 pmol

Additional Genius System Reagents

Product	Cat. No.	Size
Genius Gel Shift Kit	1635 352	20 labeling reactions and 200 gel shift reactions
Magnetic Particle Separator	1641 794	1 Separator
Anti-DIG Magnetic Particles	1641 751 1641 760	20 mg (2 ml) 100 mg (10 ml)

DNA Probes Specific for Human Chromosomes

Product	Cat. No.	Size
Y-specific, DIG-labeled	1558 196	1 µg (50 µl)
1-specific, DIG-labeled	1558 765	1 µg (50 µl)
Specific for all Human Chromosomes, DIG-labeled	1558 757	1 µg (50 µl)
17-specific, DIG-labeled	1666 452	1 µg
2-specific, DIG-labeled	1666 479	1 µg
8-specific, DIG-labeled	1666 487	1 µg
14+22-specific, DIG-labeled	1666 495	1 µg
18-specific, DIG-labeled	1666 509	1 µg
20-specific, DIG-labeled	1666 517	1 µg
X-specific, DIG-labeled	1666 525	1 µg
X-specific, fluorescein	1666 444	1 µg
17-specific, fluorescein-labeled	1666 371	1 µg
2-specific, fluorescein-labeled	1666 380	1 µg
8-specific, fluorescein-labeled	1666 398	1 µg
14+22-specific, fluorescein-labeled	1666 401	1 µg
18-specific, fluorescein-labeled	1666 428	1 µg
20-specific, fluorescein-labeled	1666 436	1 µg
X-specific, fluorescein-labeled	1666 444	1 µg
2-specific, fluorescein-labeled	1666 380	1 µg
8-specific, fluorescein-labeled	1666 398	1 µg
14+22-specific, fluorescein-labeled	1666 401	1 µg
17-specific, fluorescein-labeled	1666 371	1 µg
18-specific, fluorescein-labeled	1666 428	1 µg
20-specific, fluorescein-labeled	1666 436	1 µg
X-specific, digoxigenin-labeled	1666 525	1 µg
2-specific, digoxigenin-labeled	1666 479	1 µg
8-specific, digoxigenin-labeled	1666 487	1 µg
14+22-specific, digoxigenin-labeled	1666 495	1 µg
17-specific, digoxigenin-labeled	1666 452	1 µg
18-specific, digoxigenin-labeled	1666 509	1 µg
20-specific, digoxigenin-labeled	1666 517	1 µg

Appendix D *Trouble-Shooting Guide*

Trouble Shooting and General Hints on Good Laboratory Practice for DIG Labeling and Detection

Here we describe trouble shooting and general suggestions for good laboratory practice with the Genius System.

Work under sterile conditions

- Autoclave Genius System solutions.
- Filter-sterilize solutions containing SDS; Tween® 20 should be added to previously sterilized solutions.
- Use sterile pipette tips.
- For preparation of solutions, see Appendix B.

Use clean incubation trays

- Rigorously clean and rinse laboratory trays before each use.
- When Northern Blots are performed, use sterile glass trays for all washing and detection steps.

Membrane handling requirements

- Wear powder-free gloves when handling membranes.
- Handle membrane only on the edges and with clean forceps.

Important Hints for Success

Below, handling is described for all important steps, and the influence on sensitivity and background with the Genius System is indicated.	Influence on background	Influence on sensitivity
1. Labeling Reactions		
1.1. Incorporation of Digoxigenin-11-dUTP During PCR Amplified vector sequences can lead to nonspecific hybridization signals. Be sure to exclude vector sequences from the labeling reaction: digest the vector with a restriction enzyme such that its recognition site is as close as possible to the primer-binding sites.	+	
1.2. Random primed labeling Most important step: denaturing the probe. Be sure to denature the probe; boil for 10 min at 100°C (use a waterbath with lid), and place it immediately on ice. Cooling can be speeded up by using ice/ethanol (–7°C).		+
Do not label vector sequences. Use only fragments ≤10 kb; digest longer probes with a 4 bp cutting restriction enzyme. The smallest fragment tested so far in random primed labeling was 52 bp; the sensitivity of the reaction dropped to 1.0 pg.		+
Templates should be purified with phenol chloroform extractions prior to labeling. If a probe does not reveal a reasonable sensitivity in the direct detection assay, repurify via Elu-Tip® minicolumns (Schleicher & Schuell). This is especially necessary after fragment isolation from agarose. DNA fragments can be directly labeled after agarase treatment without further purification. After isolation with QIAEX™ Gel Extraction Kit (QIAGEN®), labeling can also be performed without problems.		+
The labeling reaction can be upscaled. This results in a higher yield of probe. A higher efficiency can also be obtained by overnight incubation.		+
Proteinase K treatment of the completed labeling reaction can enhance the sensitivity and reduce background: see page 41.	+	+
Recent experiments have shown that it is not absolutely necessary to separate the unincorporated DIG-11-dUTP nucleotides from the labeled probes.		

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	Influence on background	Influence on sensitivity
1.3. 3'-End labeling/tailing Make sure that the oligonucleotide concentration has been determined correctly. We calculate 1 OD_{260} = 33 µg oligonucleotide. The concentration of short oligonucleotides of known sequence can also be determined using A_{260}. However, the base composition of the oligonucleotide can have significant effects on absorbance. The total absorbance is the sum of the individual contributions of each base.		+
When upscaling the labeling reaction, all components have to be increased proportionally. Increasing only the oligonucleotide concentration results in insufficient labeling.		+
Sometimes it is difficult to precipitate short, labeled oligonucleotides quantitatively. Make sure that all solutions (LiCl, Glycogen dilution, ice-cold ethanol) are mixed thoroughly with the reaction mix. Instead of the Glycogen dilution, 1 µl of concentrated Glycogen can be used.		+
1.4. RNA labeling by in vitro transcription Use sterile disposable plastic ware and sterile DEPC-treated solutions.		+
Linearize template DNA for *in vitro* transcription to receive a vector-free probe and better labeling efficiency. +		+
Be sure to use the correct strand for RNA transcription if RNA on a Northern Blot is hybridized. Transcripts can also be analyzed on nondenaturating agarose gels using TAE buffer.		
For all labeling reactions it is extremely important that you check the labeling efficiency in a direct detection assay prior to hybridization.		++
2. Blotting Various methods are available for the transfer of nucleic acids from agarose gels to membranes. Most commonly used methods in order to achieve high sensitivity: capillary blot > vacuum blot > dry blot		+
2.1. Pretreatment of DNA in the agarose gel prior to transfer Depending on the size and structure of the DNA, pretreatment of the gel may be necessary for optimal transfer.		+
DNA fragments larger than 10 kb and supercoiled plasmids have to be fragmented inside the gel after electrophoresis. This can either be achieved by treating the gel with 0.25 M HCl for 5–20 min (start with 5–10 min for mammalian DNA; this can be prolonged up to 20 min, especially for plant DNA) or by UV-irradiation on a transilluminator. The latter has the advantage that only those parts of the gel containing long fragments can be exposed to UV light so that smaller fragments remain unaffected.		+
Note: For every transilluminator, irradiation conditions have to be defined empirically in a test series. With HCl treatment, be sure not to over degrade small fragments, causing them to be lost during transfer.		+
For the transfer of Mb-size fragments and supercoiled plasmids, a combination of both methods may be necessary. Conditions also have to be defined empirically.		+
2.2. Denaturation Unless alkaline transfer is performed, double-stranded DNA has to be denatured in the gel prior to transfer. This can either be accomplished with 1 M KOH (gels only) or with 0.5 M NaOH; 1.5 M NaCl (for gels and membranes; *e.g.*, Colony and plaque hybridization).		+
Subsequent neutralization can be enhanced by briefly washing the gel in distilled water after alkaline treatment.		

	Influence on background	Influence on sensitivity
2.3. Neutralization		
Especially when transfer to nitrocellulose membranes is intended, it is important to check the actual pH of the gel after neutralization. It should be below pH 9 (nylon membranes will tolerate a higher pH); otherwise, membranes will turn yellow and break during hybridization. To check the pH of the gel, lift one edge of the gel where no DNA has been loaded, press a pH stick onto it, and read the pH.		
2.4. Setting up a capillary transfer		
Nylon membranes need no pretreatment. Nitrocellulose membranes have to be pre-wetted in distilled water and 20X SSC prior to transfer. We strongly recommend the use of the Boehringer Mannheim Nylon Membranes, which are especially designed and function tested for Genius System blotting applications. Membranes have to be handled extremely carefully. Pick up membranes only with forceps at the corners; wear powder-free gloves; be careful not to leave any fingerprints.	+	+
It is important to remove air bubbles between the gel and the membrane. This can be achieved by rolling a clean pipette gently across the membrane.		+
Place Parafilm® around the gel to avoid contact of the blotting paper with the Whatman paper and the transfer buffer underneath the gel.		+
Use 20X SSC for the transfer; 10X SSC can only be applied if very large DNA fragments are transferred. Capillary blots should be performed overnight.		+
Make sure that the transfer buffer is soaked only through the gel and not around the gel. Place the paper towels so that no contact between Whatman paper underneath the gel or transfer buffer is possible. Replace the blotting paper when it becomes wet.		+
The weight applied should be in accordance with the gel size. We suggest 500 g for mini gels and a maximum of 1250 g (20 x 20 cm gels).		+
After UV fixation, carefully wash the membrane quckly in 2X SSC to avoid salt precipitates.	+	
Stain the gel with ethidium bromide to evaluate the efficiency of the transfer.		+
2.5. Fixation		
The DNA has to be fixed to the membrane prior to hybridization. This can be achieved either by UV-crosslinking (with a Stratalinker® or transilluminator) or by baking.	+	+
For most membranes, baking at +80°C for 2 h is sufficient. The Boehringer Mannheim positively charged Nylon Membrane can be baked at +120°C, but for no more than 30 min. In general, UV-crosslinking will probably be more convenient.	+	+
Especially if chemiluminescent detection is to be performed, it is important to UV-crosslink the membrane from both sides. This reduces the background coming from the opposite side of the membrane. When light is emitted during the detection, signals from both sides of the membrane will reach the X-ray film.	+	
Special hints for Northern Blots: pre-soak gels in sterile transfer buffer for 20 min prior to transfer. All types of RNA gel systems are compatible with the Genius System.		+
Ethidium bromide staining of RNA gels does not interfere with transfer. Autoclave the transfer buffer. When handling the membrane, it must be taken into account that RNases can degrade single-stranded RNA bound to a membrane.		+

Stratalinker® is a registered trademark of Stratagene.

	Influence on background	Influence on sensitivity
3.1. Prehybridization Working concentrations for Blocking Reagent during prehybridization are between 1–5%. Use a sterile Blocking Reagent stock solution (10%; see p. 86 for a description of its preparation) to prepare the prehybridization solution. Denatured fish sperm DNA or yeast RNA at a final concentration of 50 µg/ml can be added but is not necessary for most applications.	+	
Take into account that hybridization signals could eventually be blocked by cross hybridization of the target DNA with a nonspecific nucleic acid.		+
Allow the prehybridization to proceed for at least 1 h at the same temperature as the subsequent hybridization. Use a sufficient volume of prehybridization solution. If roller tubes are applied, use a minimum of 20 ml. If sealed bags or trays are used, gently shake them during the prehybridization.	+	
Do not allow the membranes to dry between prehybridization and hybridization.	++	
3.2. Hybridization with probe **Note:** It is important to carefully evaluate the correct hybridization conditions for a given probe and target.	+	+
Probe concentration is a very important factor. A probe concentration that is too high may lead to nonspecific binding of the probe to the membrane, and a probe concentration that is too low could lead to lower sensitivity. The concentrations given in this guide will work with most routine applications.	+	+
Shorter hybridization times can be applied in combination with a higher probe concentration. For important experiments, we recommend that you test the optimal probe concentration in a mock hybridization. For this purpose, pre-incubate small pieces of membrane, and then hybridize them overnight with increasing concentrations of labeled probe per ml of hybridization solution. After detection, the optimal concentration can be defined (see p. 44 for detailed description).	+	+
3.2.1. Denaturation of probe Denature DNA probes and RNA probes (secondary structures) before adding them to the hybridization solution.		+
With oligomers, denaturation is only necessary when secondary structures can be expected from the nucleotide sequence.		
Use only deionized formamide (if it is included in the hybridization solution).	+	+
3.2.2. Hybridization in roller tubes Use at least 6 ml per hybridization solution tube. This volume can be increased if required.	+	+
Monitor the hybridization temperature. Note that the temperature set on the oven is not necessarily the temperature maintained inside the roller tube. Check the temperature inside the tube before hybridization by filling the tube, for instance, with water and placing a thermometer inside the tube.	+	+

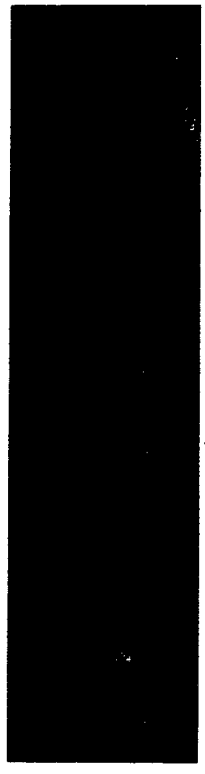
	Influence on background	Influence on sensitivity
3.2.3. Hybridization in sealed bags Use at least 2.5 ml hybridization solution per 100 cm^2 of membrane. This volume can be increased if required.	+	+
Remove all air bubbles prior to sealing.	+	+
Check the seals.		
Gently shake sealed bags in a waterbath set at the right hybridization temperature. The membrane should lie flat on the bottom of the waterbath. Uneven positioning of the membrane will cause loss of sensivity and background problems. Membranes can also be placed flat in an incubator.	+	+
The High SDS hybridization solution according to Church (main difference from other hybridization protocols is the inclusion of 7% SDS) is highly recommended for genomic applications as well as Northern Blots when DNA probes are applied. Be careful when handling this buffer because the SDS will precipitate at low temperatures. Prewarm the buffer before incubation with the membrane. Do not place this buffer on ice.	+	+
For RFLP applications, we recommend this buffer and a hybridization temperature of +55°C; no formamide is necessary.		
3.2.4. Special hints for hybridization with tailed oligonucleotides Hybridization with a tailed oligonucleotide should be performed with 0.1 µg/ml Poly (A) in the prehybridization and hybridization solution to prevent nonspecific hybridization signals. Additionally, 5 µg/ml Poly d(A) can increase blocking efficiency.		+
3.3. Special hints for Northern Blot applications Use the High SDS hybridization solution when working with DNA probes.	+	+
Use 50% formamide (deionized).	+	+
Recent results suggest that it is preferable to work with RNA probes whenever possible.		+
Work under sterile conditions.	+	+
Single-stranded RNA can be degraded by single-strand-specific RNases also when bound to membrane, and double-stranded RNA hybrids can be degraded by double-strand-specific RNases.		+
Special hint for Northern Blots when RNA probes have been used: background can be reduced by adding an RNase A wash step after the last stringent wash. Use a 100 µg/ml RNase A solution in 10 mM Tris-buffer, 5 mM EDTA, 300 mM NaCl, pH 7.5 for 30–60 min at room temperature. **Attention:** some RNase A preparations may contain double-strand-specific RNases that can degrade hybrids on the membrane. Double-strand-specific RNases can be detected by incubation with double stranded Poly RNAs or MS2 RNA followed by analysis on a denaturating agarose gel. MS2 RNA is partially double-stranded. RNase A preparations that do not contain double-strand-specific RNase activity will leave these regions unaffected. It is always necessary to compare the untreated MS2 RNA when performing such tests.	+	+

	Influence on	
	back-ground	sensi-tivity
3.4. Washes		
Optimize the washing conditions for your particular application. The conditions given in the pack insert are defined for 100% homology between target DNA and probe, and a GC content of about 50%. For genomic hybridizations, we recommend the use of 0.5X SSC for the stringency washes.	+	+
Apply vigorous shaking during the stringency washes.	+	
Prewarm the wash solutions to the appropriate temperature.	+	
Use trays rather than roller tubes for the washing steps.	+	
Do not allow the membranes to overlap or stick together during the washing steps.	+	
Use an excess volume of washing solution.	+	
4. Immunological Detection		
Use freshly washed trays.	+	
Shake membranes during the whole detection procedure (except color development).	+	
Store the antibody at +4°C. Carefully check the tube to see if a precipitate has formed. If so, remove the precipitate by a 30 sec centrifugation.	+	
Work under sterile conditions.	+	
The blocking and washing steps can be prolonged, but do not prolong the antibody reaction.	+	
The concentration of the Blocking Reagent can be increased to up to 5%.	+	
Use freshly washed trays after the antibody reaction.	+	
Prepare a fresh dilution of antibody and color substrate solutions directly before use.	+	
When working with the chemiluminescent substrates, a dilution of only 1:10,000 of the antibody is necessary.	+	
Work under absolute sterile conditions when handling the chemiluminescent substrate solution, and avoid phosphatase contamination.	+	
Allow the color reaction to develop in the dark without shaking. It is not necessary to work in the dark with the Lumigen™ PPD solution, but Lumi-Phos™ 530 and Lumi-Phos Plus are light sensitive.	+	
It is possible to switch from chemiluminescent detection to a color reaction on the same blot. Wash off the chemiluminescent substrate for 5 min with Detection buffer, and then add the color substrate. Background from the opposite side of the membrane is then excluded. It is not a problem if the higher antibody dilution has been applied previously.	+	
There are two ways to apply the chemiluminescent substrate. If many membranes have to be processed at the same time, prepare a higher volume of substrate. Wet the membranes with the substrate solution for 5 min, and let the excess liquid drip off. Do not press the membrane onto Whatman paper. Do not allow the membrane to dry.	+	
Place the membranes carefully between two sheets of plastic transparencies. Press out all air-bubbles, and seal the edges to prevent the membrane from drying. Incubate for 10 min at +37°C. This step is important to activate the unstable intermediate that is produced during the reaction. Omitting this step results in prolonged exposure times. The incubation step for 10 min at +37°C may be omitted when plaque- or colony hybridizations are performed.	+	
If diluted Lumigen PPD solution was used, filter the solution, and add sodium azide for preservation. It can be used for about 4 weeks, unless it becomes contaminated.		

	Influence on	
	back-ground	sensi-tivity
The second method is to place the membrane on a transparency. Add 500 µl of substrate solution per 100 cm^2 of membrane. Place a second transparency on the top. Spread the substrate solution carefully by pressing gently on the transparency. Incubate for 5 min at room temperature. Allow the excess volume of substrate to drip off from the transparency/membrane/transparency sandwich. Seal and preincubate for 10 min at +37°C. Then start making exposures. This method is recommended if only a few membranes are handled per week. Films with different sensitivities are available.		
A spotty background, which is sometimes observed with the chemiluminescence detection, can be suppressed by using Detection buffer without Mg^{2+} ions.	+	
All established hybridization protocols can be used with the Genius System. Buffers given in this User's Guide have been specially optimized to work with the Genius System. Denhardt's solution can be used instead of Blocking Reagent. The bands obtained are slightly fuzzy compared to those obtained with Blocking Reagent.	+	+
Hybridization signals can be intensified by the addition of 10% dextran sulphate or 6% PEG 8000, but the background will also increase. Dextran sulphate shows lot-to-lot inconsistency; with some lots, a strong background is obtained. PEG is more reliable in this respect, but we have not observed any increase in sensitivity. Only the signal intensity itself could be increased 2-3-fold.	+	+
In addition to the methods given in the pack inserts, stripping by boiling in distilled water containing 0.1% SDS can be recommended. This is especially useful when stripping northern blots because alkali treatment degrades the RNA on the blot as well as in cases where Photodigoxigenin MWM have been transferred to the membrane. If an RNA blot is rehybridized, it is preferable to use Detection buffer without Mg^{2+} ions prior to the detection. Do not use alkaline solutions for stripping RNA blots.		
It is very convenient to use DIG-labeled molecular weight markers. The markers are visualized automatically during the detection reaction, simplifying the calculation of the molecular weight of bands of interest. **Note: DIG-labeled molecular weight markers do not withstand alkali transfer and alkaline stripping.**		
To become familiar with the Genius System, start with the control reactions given in the kit.	++	++

Examples of Typical Problems

Problem 1:

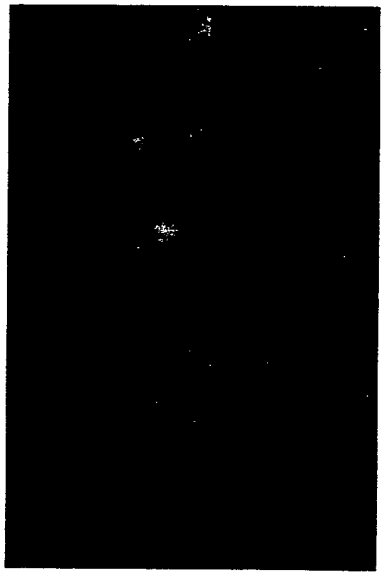


A possible cause:
An unsuitable membrane was used.

Note:
For further reasons that might cause the same problem, please refer to pages 88–94.

Recommendation:
Use the Boehringer Mannheim Nylon Membrane.

Problem 2:

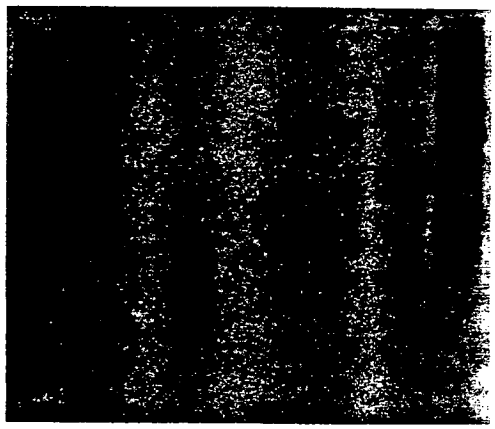


A possible cause:
A precipitate was present in the antibody preparation.

Note:
For further reasons that might cause the same problem, please refer to pages 88–94.

Recommendation:
Centrifuge the antibody for 30 sec before removing an aliquot for dilution. Be sure to use Detection buffer without Mg^{2+} ions.

Problem 3:



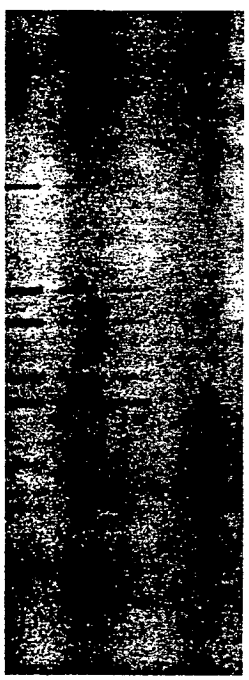
A possible cause:
A bubble occurred between the membrane and the gel during Southern or northern transfer or during hybridization.

If bands >10 kb are missing, the depurination step using 0.25 N HCl was not performed.

Note:
For further reasons that might cause the same problem, please refer to pages 88–94.

Recommendation:
Remove any air bubbles between the gel and the membrane; this can be accomplished by rolling a clean pipette gently across the membrane; also remove bubbles in the hybridization solution before the bag is sealed.

Problem 4:

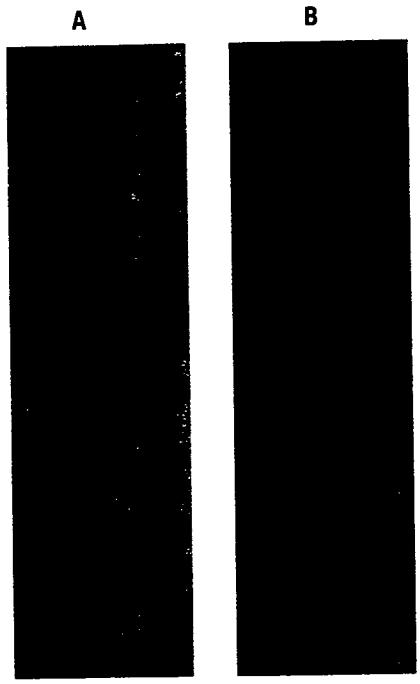


A possible cause:
- Transfer of DNA during Southern transfer was incomplete.
- The gel is mashed.
- There was a non-constant vacuum during vaccum transfer; the gel dried out.

Note:
For further reasons that might cause the same problem, please refer to pages 88–94.

Recommendation:
- Make sure that the weight is evenly distributed over the blotting paper and that the weight does not mash the gel. Check the vacuum transfer.

Problem 5:



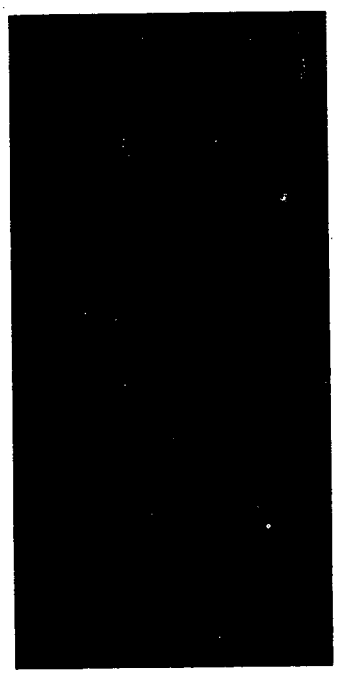
A possible cause:
(a) underexposure

(b) overexposure.

Note:
For further reasons that might cause the same problem, please refer to pages 88–94.

Recommendation:
Adjust the exposure time.

Problem 6:

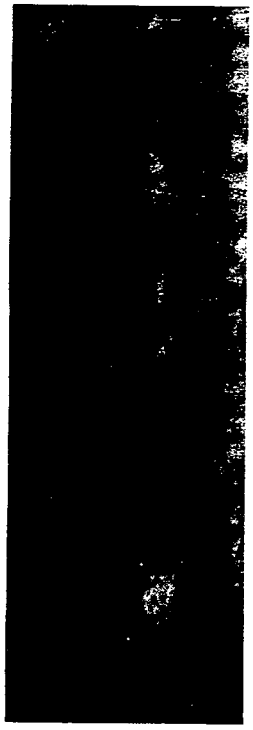


A possible cause:
The probe concentration was too high, or the template DNA was contaminated.

Note:
For further reasons that might cause the same problem please refer to pages 88–94.

Recommendation:
Perform a mock hybridization as described on page 44 to determine the highest probe concentration that can be used without resulting in high background.

Prolong the stringency wash steps (2 x 20 min) and the antibody wash steps (2 x 20 min).

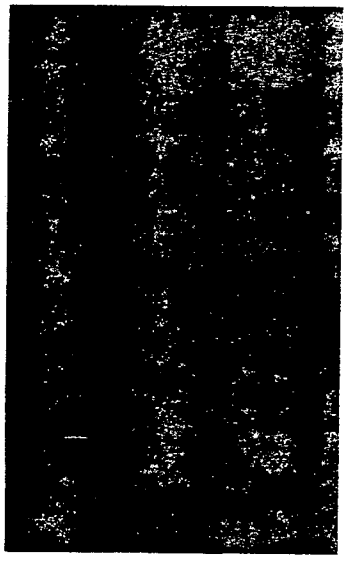


A possible cause:
Non-uniform distribution of chemiluminescent substrate during chemiluminescent detection; certain parts of the membrane are dry.

Note:
For further reasons that might cause the same problem, please refer to pages 88–94.

Recommendation:
Refer to general lab hints on page 93 where two methods for the distribution of the chemiluminescent substrate are described

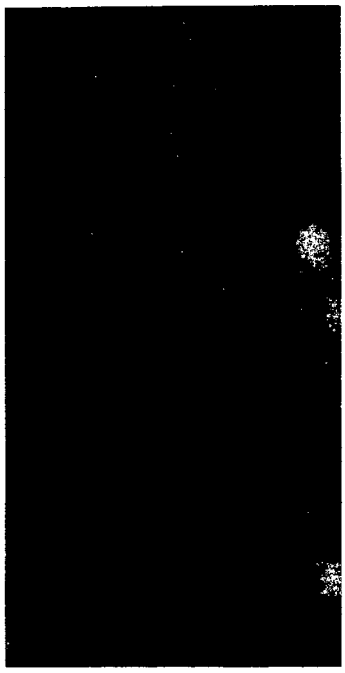
Irregular smears of background can also be caused by a crumpled hybridization bag. The bag crumples because of the heat, and this crumples the membrane in the same pattern so that the X-ray film does not have uniform contact with the membrane. To avoid this problem, make sure that the surfaces of the bag are smooth before hybridization is initiated.



A possible cause:
These outside spots on the exposed X-ray film are caused by electrostatic charge on the sealing bag.

Note:
For further reasons that might cause the same problem, please refer to pages 88–94.

Recommendation:
Wipe the surface of the sealing bag with 70% ethanol before applying the film.



A possible cause:
Distribution of probe was uneven.

Note:
For further reasons that might cause the same problem, please refer to pages 88–94.

Recommendation:

- Use at least 2.5 ml hybridization solution per 100 cm^2 membrane.
- Shake during hybridization, and make sure the bag lies flat on the bottom of the water bath.
- If a roller apparatus is used, apply at least 6 ml hybridization solution per tube.
- Do not allow the membrane to dry between prehybridization and hybridization.