CONTENTS

Promega

I. Introduction

In recent years, the complete genomes of many organisms have been sequenced, allowing genes to be identified by searching the sequence for protein-coding regions, transcription start sites and other features of a typical gene. However, this process tells us little about the gene's role in biological processes. In some cases, we can obtain clues about a gene product's function by identifying regions of sequence homology with known genes or motifs encoding well characterized protein domains. For many genes, sequence analysis alone provides insufficient insight. In these cases, knowing when and where genes are expressed often can help us gain more understanding of the gene's role in a cell. Examining the temporal and spatial patterns of gene expression can help assign function to genes involved in physiological changes (e.g., aging or fruit ripening), tumorigenesis, pathogenicity, cellular responses to stimuli and a wide variety of other cellular events.

II. Techniques to Monitor Gene Expression Levels

Many techniques have been developed to examine absolute and relative levels of gene expression (reviewed in Roth, 2002; Bartlett, 2002). Historically gene expression was monitored by Northern analysis or RNase protection assays (RPA). More recently, microarrays and PCR-based techniques, such as quantitative PCR and differential display PCR, have become popular.

A. Northern Blot Analysis

Once a gene is identified, it is useful to determine the size of the mRNA and determine if alternative splice variants of different sizes are present. This information can be used to estimate the size of the putative protein and confirm DNA sequencing data. The method used to analyze RNA in this way is Northern blot analysis, in which total or poly(A)+ mRNA is run on a denaturing agarose gel, and a specific RNA target is detected by hybridization to a labeled probe in the dried gel itself (Wang *et al.* 1991) or on a membrane (Ausubel *et al*. 2003; Sambrook and Russell, 2001). The resulting signal is proportional to the amount of target RNA in the RNA population. An example is shown in Figure 6.1, and a protocol for Northern blot analysis is provided in Section III.A.

Comparing signals from two or more cell populations or tissues reveals relative differences in gene expression levels. Absolute quantitation can be performed by comparing the signal to a standard curve generated using known amounts of an in vitro transcript corresponding to the target RNA. Analysis of housekeeping genes, genes whose expression levels are expected to remain fairly constant regardless of conditions, is often used to normalize the results, eliminating any apparent differences caused by unequal transfer of RNA to the membrane or unequal loading of RNA on the gel. Recent studies have shown that some genes thought to be suitable housekeeping genes, such as glyceraldehyde-3-phosphate dehydrogenase and β-actin, in fact may have varying levels of expression under certain circumstances (Goidin *et al*. 2001; Warrington, 2000), so take care when choosing an appropriate housekeeping gene.

The first step in Northern blot analysis is isolating pure, intact RNA from the cells or tissue of interest. Because Northern blots distinguish RNAs by size, sample integrity influences the degree to which a signal is localized in a single band (Lee and Costlow, 1987). Partially degraded RNA samples will result in the signal being smeared or distributed over several bands with an overall loss in sensitivity and possibly erroneous data interpretation. Ribonucleases (RNases) are ubiquitous enzymes that are difficult to completely and irreversibly inactivate. When isolating RNA, use precautions to avoid inadvertently introducing RNases during or after RNA isolation. Guidelines for creating an RNase-free environment and protocols for RNA isolation can be found in Section IV.A.

In Northern blot analysis, DNA, RNA and oligonucleotide probes can be used (Section IV.B), and these probes can be radiolabeled or non-radioactively labeled. The size of the target RNA, not the probe, will determine the size of the detected band, so methods that generate probes of variable lengths, such as random-primed labeling, are suitable for probe synthesis. The specific activity of the probe will determine the level of sensitivity, so we recommend using probes with high specific activities (>10^9cpm/µg), particularly for low-abundance RNAs. Labeling methods that produce probes with lower specific activities can be used to detect more abundant RNAs.

B. RNase Protection Assay

In an RNase protection assay (RPA, reviewed in Prediger, 2001), the RNA target and an RNA probe of a defined length are hybridized in solution. Following hybridization, the RNA is digested with RNases specific for single-stranded nucleic acids to remove any unhybridized, single-stranded target RNA and probe. RNases are inactivated, and the RNA is separated by denaturing polyacrylamide gel electrophoresis. The amount of intact RNA probe is proportional to the amount of target RNA in the RNA population. RPA can be used for relative and absolute quantitation of gene expression and also for mapping RNA structure, such as intron/exon boundaries and transcription start sites. An RNase protection assay protocol is given in Section III.B.

The RNase protection assay is superior to Northern blots to detect and quantify low-abundance RNAs. In Northern blots, some RNA fails to transfer or bind to the membrane, and some molecules may not be accessible for hybridization. However, this is not a concern in an RNase protection assay when hybridization takes place in solution. Thus RPA often has lower limits of detection. Also RPA can be used to distinguish and quantify RNAs with high sequence homology. When using RNase I, single-nucleotide mismatches between the probe and target RNA are sufficient for cleavage by RNases, so only perfectly matched probes will yield a protected fragment of the expected size.

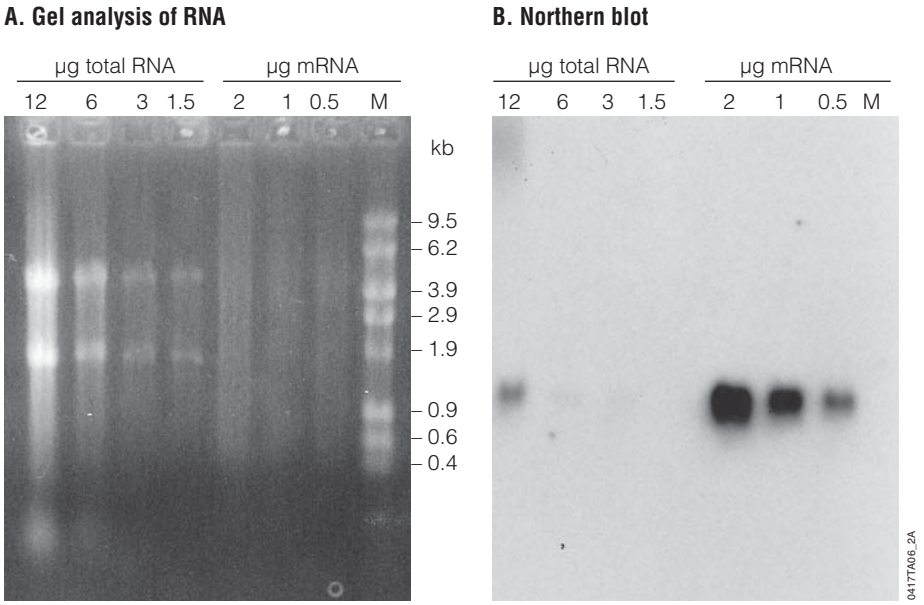

Figure 6.1. Detection of a α-1-proteinase inhibitor by Northern blot analysis. Panel A. Photograph of ethidium bromide-stained gel containing samples of total RNA isolated from mouse liver and poly(A)+ RNA (mRNA) isolated using the PolyATtract® System 1000 (Cat.# Z5400). Amounts of RNA loaded are given at the top of the lanes. Lane M contains RNA Markers (Cat.# G3191), the sizes of which are given along the side of the gel. **Panel B.** Northern blot of the gel in Panel A. The probe used was an alkaline phosphatase-oligonucleotide conjugate specific to the α-1-proteinase inhibitor mRNA, which is an abundant message in liver. The probe was prepared and analyzed by a chemiluminescent detection method. The film was exposed to X-ray film for 2 hours at 37°C.

The antisense RNA probes used in RPA are generated by in vitro transcription of a DNA template with a defined endpoint and are typically in the range of 50–600 nucleotides. The use of RNA probes that include additional sequences not homologous to the target RNA allows the protected fragment to be distinguished from the full-length probe. RNA probes typically are used instead of DNA probes due to the ease of generating single-stranded RNA probes and the reproducibility and reliability of RNA:RNA duplex digestion with RNases (Ausubel *et al*. 2003). For the most sensitive detection of low-abundance RNAs, we recommend using probes with high specific activities. A protocol for generating single-stranded, high-specific-activity RNA probes that are suitable for RNase protection assays can be found in the *Riboprobe® in vitro Transcription Systems Technical Manual* #TM016.

C. Microarrays

One of the biggest limitations of Northern blot analysis and RNase protection assays is the inability to analyze more than a few genes at a time. The use of microarrays overcomes this limitation and enables researchers to examine thousands of genes in one experiment, giving scientists a more comprehensive view of the genes involved in a specific cellular event.

The microarray process can be divided into two main parts. First is the printing of known gene sequences onto glass slides or other solid support followed by hybridization of fluorescently labeled cDNA (containing the unknown sequences to be interrogated) to the known genes immobilized on the glass slide. After hybridization, arrays are scanned using a fluorescent microarray scanner. Analyzing the relative fluorescent intensity of different genes provides a measure of the differences in gene expression.

One method of creating arrays is by immobilizing PCR products onto activated glass surfaces. Typically, these probes are generated by PCR or RT-PCR and cloned into a plasmid vector to create a library of 10,000 or more clones. This plasmid library is stored in *E. coli*. Whenever the researcher needs to make a new array, the *E. coli* clones are grown, plasmids are isolated and the cloned genes are amplified with primers common to the plasmid backbone. These amplified products tend to be longer sequences, typically in the range of 100–1,000 bases. A robot is then used to print thousands of the amplified clones in an array of 50–200µm spots on a specially prepared glass slide or other suitable support.

DNA arrays also can be generated by immobilizing presynthesized oligonucleotides onto prepared glass slides. In this case, representative gene sequences are manufactured and prepared using standard oligonucleotide synthesis and purification methods. These synthesized gene sequences are complementary to the genes of interest and tend to be shorter sequences in the range of 25–70 nucleotides. Alternatively, immobilized oligos can be chemically synthesized in situ on the surface of the slide. In situ oligonucleotide synthesis involves the consecutive addition of the appropriate nucleotides to the spots on the microarray; spots not receiving a nucleotide are protected during each stage of the process using physical or virtual masks.

In expression profiling microarray experiments, the RNA templates used are representative of the transcription profile of the cells or tissues under study. First RNA is isolated from the cell populations or tissues to be compared. Each RNA sample then is used as a template to generate fluorescently labeled cDNA via reverse transcription. Fluorescent labeling of the cDNA can be accomplished by either direct-labeling or indirect-labeling methods. During direct labeling, fluorescently modified nucleotides (e.g., Cy®3- or Cy®5-dCTP) are incorporated directly into the cDNA during the reverse transcription. Alternatively, indirect labeling can be achieved by incorporating aminoallyl-modified nucleotides during cDNA synthesis, then conjugating an N-hydroxysuccinimide (NHS)-ester dye to the aminoallyl-modified cDNA after the reverse transcription reaction is complete.

To perform differential gene expression analysis, cDNA pools generated from different RNA samples are labeled in different colors. For example, when comparing RNA from tumor and normal tissue samples, the cDNA generated from the tumor RNA can be labeled with Cy®3, while the cDNA generated from the normal RNA sample can be labeled with Cy®5. The resulting labeled cDNA is purified to remove unincorporated nucleotides, free dye and residual RNA. Following purification, the labeled cDNA samples are combined, then hybridized to the microarray. The microarray is scanned post-hybridization using a fluorescent microarray scanner (Figure 6.2). The fluorescent intensity of each spot indicates the level of expression for that gene; bright spots correspond to strongly expressed genes, while dim spots indicate weak expression. Multiple targets labeled in different dye colors can be analyzed simultaneously to determine which genes are differentially expressed.

Once the images are obtained, the raw data must be analyzed (reviewed in Dharmadi and Gonzalez, 2004; Roth, 2002). First, the background fluorescence must be subtracted from the fluorescence of each spot. The data then are normalized to a control sequence, such as an exogenously added RNA (Schena *et al*. 1995) or a housekeeping gene to account for any nonspecific hybridization, array imperfections or variability in the array setup, cDNA labeling, hybridization or washing. Care must be taken when choosing an appropriate housekeeping gene; recent studies have shown that some genes thought to be suitable housekeeping genes, such as glyceraldehyde-3-phosphate dehydrogenase and β-actin, may in fact have varying levels of expression under certain circumstances (Goidin *et al*. 2001; Warrington, 2000). Data normalization allows the results of multiple arrays to be compared.

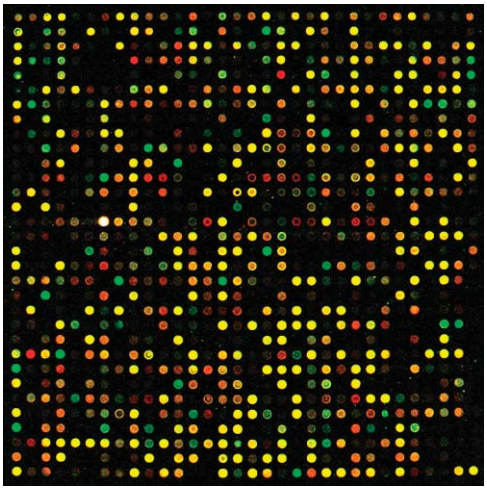

Figure 6.2. Cy®3- and Cy®5-labeled cDNA hybridized to a DNA microarray. Cy®3-labeled cDNA from 293T mRNA (green) and Cy®5-labeled cDNA from HeLa mRNA (red) were hybridized to custom 4K arrays provided by Corning Incorporated. Data were acquired using a Genepix® 4000B scanner (Axon Instruments, Inc.). A representative subgrid is shown. The scanner generates separate data images for green (532nm) and red (635nm) wavelengths. By overlaying the red and green data images, you can view the differential expression of various genes. Green spots indicate expression in the 293T sample, while red spots indicate expression in the HeLa sample. Yellow spots indicate genes that were expressed in both samples. The fluorescence intensity of each color at each spot indicates the level of expression of that gene in each sample.

Table 6.1. Expression Analysis Techniques.

Technique	Nucleic Acid Labeled	Advantages
Northern blot analysis	RNA or DNA probe	• provides information about the quantity and size of the RNA target • can be used to determine whether splice variants of different sizes exist
RNase Protection Assay	RNA probe	• offers superior quantitation of RNA, allowing more sensitive analysis of low-abundance RNAs • can be used to map RNA structure • can distinguish and quantify RNAs with high sequence homology
Microarrays	cDNA	• allows analysis of thousands of genes in a single experiment • can simultaneously analyze multiple targets labeled with different dyes

Additional Resources for Microarrays

Promega Publications

High-throughput DNA fragment purification using the MagneSil® automated 384-well clean-up systems

Introducing the Wizard® SV 96 PCR Clean-Up System

Indirect fluorescent labeling of microarray targets using ImProm-II™ Reverse Transcriptase

RNA Analysis Notebook

Online Tools

Microarray FAQ

D. Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) is a common method to quantify target mRNA levels in a sample. To perform qRT-PCR, RNA samples are reverse transcribed to produce cDNA, then the cDNA is quantified by quantitative PCR. qPCR can be performed as an end-point assay or, more commonly, in real time using fluorescently labeled oligonucleotide probes or primers or DNA-binding fluorescent dyes such as SYBR® Green. For more information about qPCR, see the Nucleic Acid Amplification chapter.

III. Expression Analysis Protocols

Tips to create an RNase-free environment and choose an RNA isolation protocol can be found in Section IV.A. A discussion of RNA and DNA labeling techniques can be found in Section IV.B.

A. Northern Blot Analysis Protocol

Agarose/Formaldehyde Gel Electrophoresis of RNA
Materials Required:
(see Composition of Solutions section)

- agarose
- MOPS 5X and 1X buffer
- 37% formaldehyde
- DEPC-treated water
- RNA markers (optional)
- RNA sample buffer
- RNA loading buffer

1. Prepare a 1% gel by combining the following components (sufficient for a 280ml gel):

MOPS 5X buffer	56.0ml
DEPC-treated water	174.0ml
agarose	2.79g

   Mix well, and boil to dissolve the agarose.

2. Cool to 55°C, and add 50ml of 37% formaldehyde. Mix thoroughly and pour a 0.5–1.0cm thick gel. Allow to solidify.

   Note: Do not add ethidium bromide to the gel or running buffer because it will decrease the efficiency of RNA transfer to the membrane.

3. Prepare the RNA samples by mixing 1 part RNA with 2 parts RNA sample buffer up to a total volume of 10–30µl, depending upon the thickness of the gel. Heat samples at 65°C for 5 minutes, cool to room temperature, then add 2µl of RNA loading buffer.

   Note: We recommend loading 0.2–10µg of total RNA for a high-abundance message. The amount of RNA required depends upon transcript abundance. Up to 30µg of total RNA per well can be used. For rare messages, we recommend isolating poly(A)+ RNA and loading at least 3µg per well.

4. Prerun the gel for 10 minutes in MOPS 1X buffer prior to loading the samples. Load samples, then run the gel at 4–5V/cm. Continue electrophoresis until the bromophenol blue has migrated at least 10cm from the wells.

   Note: RNA markers should be run along with the RNA samples of interest if size determination is an important factor in the experiment. If desired, load duplicate sets of samples and excise one set after electrophoresis for ethidium bromide staining. The integrity of the samples can be confirmed by the presence of the 28S and 18S ribosomal RNA bands.

Transfer of RNA to Membranes
Many different setups to transfer nucleic acids to membranes exist. The following procedure is a general guideline for upward capillary transfer of RNA to a membrane and may not be optimal for all types and brands of hybridization membranes. Follow the manufacturer's

recommendations for optimal results. An alternative protocol for downward capillary transfer can be found in Sambrook and Russell, 2001.

Materials Required:
(see Composition of Solutions section)
- SSC: 20X, 5X
- NaOH, 0.05N (optional)
- DEPC-treated water
- nylon hybridization membrane
- Whatman® 3MM paper or equivalent
- 12 blotting pads per Northern transfer procedure
- Stratalinker® UV Crosslinker or equivalent, or vacuum oven

1. Soak the gel several times in DEPC-treated water at room temperature to remove the formaldehyde.

   Note: Use care in handling the gel because it will be more fragile than standard agarose gels.

2. **Optional:** Soak the gel in 0.05N NaOH for 20 minutes at room temperature to partially hydrolyze the RNA. Rinse the gel in DEPC-treated water.

   Note: This step is beneficial if the RNA to be transferred is >2.5kb in length, the gel is >1% agarose or the gel is >5mm thick. The partial hydrolysis of the RNA will improve the transfer efficiency of high-molecular-weight RNA from the gel.

3. Soak the gel in 20X SSC for 45 minutes at room temperature.

4. While the gel is soaking, cut 2 pieces of Whatman® 3MM filter paper and 1 piece of hybridization membrane to the exact size of the gel.

   Note: Wear gloves, and handle the hybridization membrane by the edges using forceps to avoid background artifacts. Prewet the hybridization membrane in distilled water, and soak it in 20X SSC until needed.

5. Stack 4 blotting pads, and completely saturate them in 20X SSC.

   Note: We have noted that sponges can be a source of background in biotin-streptavidin systems. We highly recommend using only blotting pads for the transfer process. These work exceptionally well in providing even and efficient transfers without background.

6. Place the presoaked (completely saturated) blotting pads in a clean glass or plastic container. Wet 2 pieces of Whatman® 3MM paper in 20X SSC. Place one of the moistened pieces of filter paper on top of the blotting pads.

7. Place the gel on top of the filter paper with the bottom side of the gel up. Surround the gel with cut pieces of Parafilm® to prevent short-circuit wicking of the transfer solution.

8. Using gloved hands, place the membrane on top of the gel. Do not move the membrane once it has been placed on the gel. Remove any air bubbles by gently rubbing a gloved hand over the back of the membrane or by gently rolling a clean 25ml pipette over the surface of the membrane.

9. Place the second piece of Whatman® 3MM filter paper (prewet with 20X SSC) on top of the hybridization membrane.

   Note: Keep the membrane out of direct contact with the blotting pads to reduce background problems.

10. Place the remaining 8 (dry) precut blotting pads on top of the Whatman® 3MM filter paper.

11. Place a glass plate over the top of the blotting pads along with a full 250ml bottle as a weight. Allow the transfer to proceed for 1–16 hours at room temperature.

    Note: Transfer times should be about 10 minutes per millimeter of gel thickness. Transfer under these conditions ensures complete denaturation of RNA during transfer without significant RNA degradation. The rapid-transfer process ensures minimum gel compression and high transfer efficiency.

12. After the transfer is complete, carefully disassemble the blotting apparatus, leaving the hybridization membrane in contact with the gel. It is usually convenient at this time to mark the back of the membrane with a pencil to indicate which is the RNA side.

    Note: The transferred gel can be stained with 0.02% methylene blue in 0.3M sodium acetate (pH 5.2) and destained with water to determine if the transfer was complete. Alternatively, the gel can be stained with ethidium bromide and visualized under ultraviolet light.

13. Carefully remove the membrane, and wash the blot once in 5X SSC for 5 minutes at room temperature. Remove any pieces of agarose that may be stuck to the membrane with a gloved hand.

14. Allow the membrane to dry for 5 minutes. For nylon membranes, UV-crosslink the RNA to the membrane (RNA side up). For a Stratalinker® UV Crosslinker, we routinely irradiate at the recommended 120 millijoules (using the Auto-Crosslink setting). For nitrocellulose membranes, bake the membrane for 2 hours at 80°C in a vacuum oven between two pieces of Whatman® 3MM filter paper. In place of UV irradiation, nylon membranes also can be baked for 2 hours in a vacuum oven; however, the hybridization signal is decreased.

15. If it is not convenient to perform the prehybridization at this time, the crosslinked membrane may be placed between two sheets of Whatman® 3MM paper, wrapped in aluminum foil and stored at room temperature.

Hybridization of Probe
Materials Required:
(see Composition of Solutions section)

- radiolabeled DNA or RNA probe (see Section IV.B)
- heat-sealable bags or containers
- boiling water bath
- water bath, preheated to 42°C
- water bath, preheated to 68°C
- prehybridization/hybridization solution, prewarmed to 42°C
- SSC: 5X, 2X
- stringency wash solution I
- stringency wash solution II, preheated to 68°C

1. Wet the membrane completely in 5X SSC for 2 minutes.
2. Place the membrane in a heat-sealable bag or a sealable container.

   Note: Ensure that the container will not leak if a radioactively labeled probe is to be used.
3. Add 0.2ml of prehybridization/hybridization solution (preheated to 42°C) per square centimeter of the membrane. Seal the container. Incubate at 42°C for 1–2 hours.

   Note: If a sealable bag is used, leave room between the membrane and the seal to allow a corner of the bag to be removed when the probe is added in Step 6.
4. Denature the probe in a boiling water bath for 5 minutes, and quick-chill on crushed ice.
5. Prepare the hybridization solution by adding an appropriate amount of denatured, labeled probe to fresh prehybridization/hybridization solution (0.2ml per square centimeter of the membrane, preheated to 42°C), and mix.

   Note: For a radioactively labeled probe with a specific activity of 10^8cpm/µg, add the probe to a concentration of 10ng/ml. For a probe with a specific activity of >10^9cpm/µg, add the probe to a concentration of 2ng/ml. For a non-radioactively labeled probe, add probe to a final concentration of 10–100ng/ml.
6. Open the container or resealable bag (by cutting off a corner), and decant the prehybridization/hybridization solution. Immediately add the hybridization solution, and reseal the container or bag. Incubate at 42°C overnight.

   Note: It is possible to add the denatured probe directly to the prehybridization/hybridization solution in the container or bag, reseal and mix.
7. After hybridization, transfer the blots immediately to 300ml of stringency wash solution I. Wash with gentle shaking for 5 minutes at room temperature. Repeat for a second wash.

   Note: For oligonucleotide probes, reduce the wash times to 1–2 minutes.
8. Pour off the stringency wash solution I, and immediately add 300ml of stringency wash solution II prewarmed to 68°C. Wash with gentle shaking for 15 minutes at 68°C. Repeat for a second wash.

   Note: This is a high-stringency wash. For a moderate-stringency wash, reduce the temperature of the wash to 42°C. For a low-stringency wash, increase the SSC concentration to 0.2X and wash twice for 5 minutes at room temperature. Conditions that may require lower stringency washes include the use of probes with low melting temperatures (e.g., short probes or AU- or AT-rich probes) and probes that do not have high homology with the RNA target (e.g., degenerate probes or probes derived from related gene sequences).
9. Wash the blots with 300ml of 2X SSC for 10 minutes at room temperature with gentle shaking to remove excess SDS.
10. Wrap the blot in plastic wrap, and perform autoradiography if the probe is radioactively labeled. For a non-radioactive probe, follow the appropriate detection protocol. Do not allow the blot to dry out, especially if it is to be probed again.

    Note: Nylon blots can be stripped of probe and rehybridized. To strip a nylon membrane, incubate the blot for 1–2 hours in 10mM Tris-HCl (pH 7.4), 0.2% SDS preheated to 70–75°C or in 50% deionized formamide, 0.1X SSC, 0.1% SDS preheated to 68°C (Sambrook and Russell, 2001).

B. RNase Protection Assay Protocol

The following protocol has been optimized for use with RNase ONE™ Ribonuclease. Further details on ribonuclease protection assays are provided in Ausubel *et al*. 2003; Sambrook and Russell, 2001; Melton *et al*. 1984.

Many factors, including Na^+ concentration, probe sequences, reaction temperature, annealing temperature and RNase ONE™ Ribonuclease concentration, affect the detection of complementary RNAs by this method. We recommend performing titration experiments to optimize conditions specific for your sequences. A good discussion of these experimental variables can be found elsewhere (Lee and Costlow, 1987). When mapping AU-rich regions (>75% A + U), such as the regions found at the 3′-end of oncogenes, we recommend the alternative procedures found in Brewer and Ross, 1990, and Brewer *et al*. 1992. In addition, the use of an RNase that does not cut at A or U residues (e.g., RNase T1) can reduce the background due to cleavage in AU-rich regions, as these duplex regions "breathe" (spontaneously and temporarily denature to form single-stranded regions).

Experimental Considerations
RNA Probe

Prepare a labeled RNA probe by in vitro transcription as described in Section IV.B. Remove the template DNA by RQ1 RNase-Free DNase digestion, phenol:chloroform extraction and ethanol precipitation to prevent background hybridization. Gel purification is not a substitute for DNase treatment, as residual DNA fragments can copurify with the probe and compete for hybridization with the RNAs to be analyzed.

Longer probes (>300bp) may require gel purification after DNase treatment because the probe may contain shorter RNA species due to sequence-specific pausing or premature termination of the bacteriophage polymerase before completion of the transcript. Short probes (150–300bp) can often be used without gel purification. Purify the probe from an acrylamide gel by incubating the gel slice in 0.5M ammonium acetate, 1mM EDTA, 0.2% SDS at 37°C for 1–2 hours or overnight (overnight incubation will produce higher yields).

It is important to use a molar excess of the probe. When using a radioactively labeled probe, use only ^{32}P-labeled probes with a minimum specific activity of 1–3 × 10^8cpm/µg. We recommend 1–5 × 10^5cpm of probe per reaction. The probe should be stored at –70°C and used within 3 days of preparation to minimize background.

RNA Sample

The RNA should have a minimum $A_{260/280}$ ratio of 1.9. If the $A_{260/280}$ ratio of your sample is less than 1.9, repeat the extraction and ethanol precipitation. Use 5–10µg of total RNA to detect more abundant sequences. Use 30–40µg of total RNA or 500ng–1µg of poly(A)+ RNA to detect rare sequences.

The target RNA must be intact. Check the integrity of total RNA by separating the RNA on a denaturing agarose gel and staining with ethidium bromide. For mammalian RNA, the staining intensity of the 28S ribosomal RNA band should be approximately twice that of the 18S ribosomal RNA band if the RNA is undegraded.

Amount of RNase

The RNases commonly used in an RPA are RNase I , RNase T1 or a combination of RNase T1 and RNase A. RNase I cleaves after all four ribonucleotides, RNase T1 cleaves after G residues and RNase A cleaves after A and U residues. For most efficient cleavage of the single-stranded regions immediately adjacent to the double-stranded RNA, we recommend RNase I (RNase ONE™ Ribonuclease, Cat.# M4261). To map sequences, use 1–10u RNase ONE™ Ribonuclease per 10µg of total RNA or 0.1–1u of RNase ONE™ Ribonuclease per 1µg of poly(A)+ RNA. To analyze sequences containing a single-base mismatch, it may be necessary to add 40 times more RNase ONE™ Ribonuclease to the reaction.

Annealing and Digestion Temperatures

Most samples anneal efficiently at 37–45°C in hybridization buffer. RNase ONE™ Ribonuclease digestion works most efficiently at 20–37°C (Brewer *et al*. 1992). Annealing at lower temperatures may be required to maintain hybrids of AU-rich sequences during RNase ONE™ Ribonuclease digestion.

Protocol

Materials Required:

(see Composition of Solutions section)

- RPA hybridization buffer
- RNase digestion buffer
- ^{32}P-labeled RNA probe with a minimum specific activity of 1–3 × 10^8cpm/µg
- purified total RNA or poly(A)+ RNA from the tissue or cells of interest
- RNase-ONE™ Ribonuclease (Cat.# M4261)
- ice-cold 100% ethanol
- 3.0M ammonium acetate (pH 5.2)
- ice-cold 70% ethanol
- 20% w/v SDS
- 20mg/ml proteinase K
- phenol:chloroform:isoamyl alcohol
- carrier transfer RNA (tRNA), 10mg/ml
- RPA loading dye

1. Combine sample RNA and RNA probe in an RNase-free 1.5ml microcentrifuge tube. Include a sample containing 10µg of tRNA and the probe as a control for background hybridization and complete digestion.

   Note: Use 5–10µg of total RNA to detect more abundant sequences. Use 30–40µg of total RNA or 500ng–1µg of poly(A)+ RNA to detect rare sequences.

2. Ethanol precipitate the samples by adding 0.1 volume of 3.0M ammonium acetate (pH 5.2) and 2.5 volumes of ice-cold 100% ethanol. Mix and incubate at –20°C for 30 minutes. Centrifuge at maximum speed in a microcentrifuge for 15 minutes at 4°C.

   Note: Coprecipitation of the probe and sample ensures that the subsequent annealing is reproducible. The probe and sample may be added without ethanol precipitation to the hybridization buffer, but the additional volume will alter the final hybridization conditions. Consider how possible changes in stringency will affect the results. Futhermore, the omission of the ethanol precipitation step makes this direct approach more susceptible to inhibitors and contaminants in the RNA sample or probe. However, some RNA may be lost during the precipitation step.

3. Remove the supernatant. Wash the pellet with 1ml of ice-cold 70% ethanol, centrifuge briefly in a microcentrifuge and remove all ethanol. Dry the sample at room temperature for 5 minutes. The tubes may be monitored with a Geiger counter to avoid pellet loss.

4. Resuspend the pellets completely in 30µl of RPA hybridization buffer.

5. Incubate the samples at 85°C for 5 minutes to denature the RNAs, then incubate each sample for 2–16 hours at an appropriate annealing temperature. See Annealing and Digestion Temperature in Experimental Considerations.

6. Add 300µl of RNase digestion buffer and the appropriate amount of RNase ONE™ Ribonuclease (see Amount of RNase in Experimental Considerations). Incubate the samples for 30–60 minutes at 20–37°C.

7. Stop the reaction as follows: Add 10µl of 20% w/v SDS and 2.5µl of 20mg/ml proteinase K. Incubate for 15 minutes at 37°C. Extract once with phenol:chloroform:isoamyl alcohol, and remove the aqueous phase to a clean microcentrifuge tube containing 1µl of 10mg/ml carrier tRNA .

   Note: Alternatively, stop the reaction by adding 3.3µl of 10% SDS and 20µg of carrier tRNA and mixing.

8. Add 825µl of ice-cold 100% ethanol, and chill at –20°C for 30 minutes, then centrifuge at maximum speed in a microcentrifuge for 15 minutes at 4°C to pellet the RNA. Carefully remove the supernatant. Wash the pellet using 300µl of 70% ethanol and dry.

9. Resuspend the pellet by vortexing in 10µl of RPA loading dye.

10. Incubate at 85°C for 5 minutes to denature the RNA, and place on ice. Vortex and centrifuge briefly in a microcentrifuge.

11. Resolve the samples on a 5–8% polyacrylamide/7M urea gel, and detect the fragments by autoradiography.

C. Microarray Protocol

Promega offers two systems for labeling cDNA for microarray analysis. The ChipShot™ Direct Labeling and Clean-Up System (Cat.# Z4100) provides an efficient, reproducible method to generate fluorescent cDNA by direct incorporation of Cy®-labeled nucleotides in a reverse transcription reaction. The ChipShot™ Direct Labeling System protocol is optimized to account for differences in the efficiency of incorporating Cy®3- vs Cy®5-labeled dCTP, resulting in robust synthesis of labeled cDNA with both Cy®-labeled nucleotides.

The ChipShot™ Indirect Labeling and Clean-Up System (Cat.# Z4000) provides reagents and protocols to generate fluorescent cDNA without the use of Cy®-labeled nucleotides. Indirect labeling is achieved by incorporating aminoallyl-modified nucleotides during cDNA synthesis, followed by conjugation of a CyDye® NHS-ester dye to the aminoallyl-modified cDNA after the reverse transcription reaction is complete.

Both the ChipShot™ Direct Labeling and ChipShot™ Indirect Labeling Systems are optimized for use of total RNA or poly(A)+ mRNA as templates for cDNA synthesis. When total RNA is used, only 5µg of RNA template is required to generate sufficient labeled cDNA for hybridization to a minimum of two or three full 22 × 50mm arrays. Compared to many other commercially available systems that require 10–25µg of total RNA template, the ChipShot™ Labeling Systems allow users to conserve limited RNA template and increase the number of replicates performed. When poly(A)+ mRNA is used as the template for cDNA synthesis, only 1.5µg is required. The PolyATtract® mRNA Isolation System provides an efficient method for isolating mRNA for use in cDNA-labeling experiments.

IV. Related Protocols

A. RNA Isolation

Successful analysis of gene expression by RPA, Northern analysis, RT-PCR or microarray analysis requires pure, intact RNA. The RNA must be free of DNA and potential inhibitors that can interfere with labeling or hybridization. Successful isolation of intact RNA requires four essential steps: i) effective disruption of cells or tissue; ii) denaturation of nucleoprotein complexes; iii) inactivation of endogenous RNase activity; and iv) removal of contaminating DNA and proteins. Most important is the immediate inactivation of endogenous RNase activity, which is released from membrane-bound organelles upon cell disruption, to minimize RNA degradation. RNA is notoriously susceptible to degradation, and special care is required for its isolation. All methods of RNA isolation use strong denaturants to inhibit endogenous RNases. RNases, in contrast to deoxyribonucleases (DNases), are difficult to inactivate because they do not require cofactors and are heat-stable, refolding following heat denaturation. Some tissues such as pancreas and spleen are naturally rich in RNases, while other tissues such as liver are low in RNases.

About RNA

RNA is found in the nucleus, cytoplasm and mitochondria of eukaryotic cells. Total cytoplasmic RNA consists of ribosomal RNA (rRNA), transfer RNA (tRNA), messenger RNA (mRNA) and other small species of RNA. Heteronuclear RNA (hnRNA), the precursor of mRNA, is present in the nucleus. Only 1–2% of the total RNA in eukaryotic cells is mRNA; the majority of total RNA consists of rRNA (Ausubel *et al*. 2003). The amount of mRNA in mammalian cells has been estimated at approximately 500,000 mRNA molecules per cell (Ausubel *et al*. 2003).

With rare exceptions, all species of eukaryotic mRNAs are polyadenylated. Some viral RNAs also are polyadenylated and reside in the cytoplasm or mitochondria. In contrast, bacterial mRNAs are generally not polyadenylated, although some bacterial RNA is polyadenylated (Gopalakrishna *et al*. 1981).

Polyadenylic acid is added in the nucleus to the free 3′-OH of hnRNA following cleavage and is required for mRNA transport into the cytoplasm (Huang and Carmichael, 1996). The typical length of poly(A) addition is 200 bases in mammalian cells (Huang and Carmichael, 1996), while

mRNA isolated from plant chloroplasts contains poly(A)+ tails of only approximately 20 bases (Murillo *et al*. 1995). The length of the poly(A)+ tail can vary during the life of the message and decreases with age for a given message (Lewin, 1980). In higher eukaryotic cells, changes in polyadenylation function to control translation in the cytoplasm and to stabilize the message during early development (Winkles and Grainger, 1985; Pfarr *et al*. 1986; Salles *et al*. 1992).

The steady state level of mRNA in the cytoplasm is a combination of three factors: the rate of production, the rate of degradation and the rate of transport from the nucleus. The half-life of mRNAs in mammalian cells ranges from hours to days (Ausubel *et al*. 2003), while in yeast the half-life is 4–45 minutes (Herrick *et al*. 1990). In bacteria, the half-life is much shorter, typically a few minutes (Selinger *et al*. 2003).

Creating a Ribonuclease-Free Environment

Ribonucleases are extremely difficult to inactivate. Great care should be taken to avoid inadvertently introducing RNases into the RNA preparation during or after isolation. This is especially important if the starting material has been difficult to obtain or is irreplaceable. The following notes may be helpful in preventing the accidental contamination of the sample with RNases, allowing the isolation of full-length RNA.

- Two of the most common sources of RNase contamination are the researcher's hands and bacteria or molds, which may be present on airborne dust particles or laboratory glassware. To prevent contamination from these sources, sterile technique should be employed when handling any reagents used for RNA isolation or analysis. Gloves should be worn at all times.
- Whenever possible, use sterile, disposable plasticware when handling RNA. These materials are generally RNase-free and do not require pretreatment to inactivate RNases.
- Nondisposable glassware and plasticware should be treated before use to ensure that it is RNase-free. Glassware should be baked at 200°C overnight. Plasticware should be thoroughly rinsed before use with 0.1N NaOH/1mM EDTA, then with diethyl pyrocarbonate (DEPC)-treated water. Equipment that cannot be conveniently treated with DEPC can be treated with an RNase decontamination solution, such as RNase*Zap* ® (Ambion).

  Note: COREX® tubes should be rendered RNase-free by treatment with DEPC and not by baking; baking will increase the failure rate of this type of tube during centrifugation. COREX® tubes should be treated with 0.05% DEPC overnight at room temperature, then autoclaved for 30 minutes to remove any trace of DEPC.
- Autoclaving alone is not sufficient to inactivate RNases. Solutions supplied by the researcher should be treated with 0.05% DEPC overnight at room temperature, then autoclaved for 30 minutes to remove any trace of DEPC. Alternatively, RNases in a reaction can be inactivated by adding RNasin® Ribonuclease Inhibitor, which inhibits a broad spectrum of RNases, including RNase A, RNase B, RNase C and human placental RNase, and is active over a broad pH range (pH 5.5–9).

  Note: Tris buffers and any chemicals containing primary amine groups cannot be treated with DEPC. Use caution when weighing out Tris to avoid RNase contamination, and use DEPC-treated water and glassware when preparing Tris buffers.
- While most sources of fresh deionized water are free of contaminating RNase activity, deionized water is a potential source of RNase activity. If degradation of the target or probe RNA occurs, it may be necessary to test the laboratory's water source for RNase activity.
- We recommend that chemicals for use in RNA isolation and analysis be reserved separately from those for other uses. Wear gloves when handling labware and reagents, and use only baked spatulas and untouched weigh boats or weigh paper.

Choosing an RNA Isolation Protocol

One of the first considerations when deciding on an RNA purification protocol is whether you will be using total RNA or poly(A)+ RNA for your application. The source of RNA, type of RNA to be purified, relative abundance of the RNA, sample size and convenience of the isolation procedure are all factors that also must be considered. For valuable tissue samples, we suggest that a portion of each sample be reserved at –70°C in the event that loss of a sample occurs during RNA purification. Promega offers both total RNA isolation systems (SV Total RNA Isolation System, PureYield™ RNA Midiprep System and MagneSil® Total RNA mini-Isolation System) and poly(A)+ RNA isolation systems (PolyATtract® System 1000) that yield clean, intact RNA from a variety of cell and tissue types.

Additional Resources for RNA Isolation

Technical Bulletins and Manuals

TM048	*SV Total RNA Isolation System Technical Manual*
TB294	*SV 96 Total RNA Isolation System Technical Bulletin*
TM021	*PolyATtract ® mRNA Isolation Systems Technical Manual*
TM228	*PolyATtract ® System 1000 Technical Manual*

Promega Publications

RNA purification kit comparison: Yield, quality and real-time RT-PCR performance

Cleanup of TRIzol® reagent-purified total RNA using the PureYield™ RNA Midiprep System

PureYield™ RNA Midiprep System: Isolating pure total RNA without DNase

MagneSil® Total RNA mini-Isolation System

Quantitative, real-time RT-PCR expression using the SV 96 Total RNA Isolation System

High-throughput purification using the SV 96 Total RNA Isolation System

Isolation of RNA from plant, yeast and bacteria

RNA purification: A rapid and versatile protocol for the isolation of total RNA

Technically speaking: Tips for working with RNA and troubleshooting downstream applications

RNA Analysis Notebook

Measuring gene expression from mammalian brain tissue

Total RNA Isolation Using the SV Total RNA Isolation System

The SV Total RNA Isolation System provides a fast and simple technique to purify intact total RNA from tissues, cultured cells and white blood cells in as little as one hour, depending on the number of samples to be processed. Up to 60mg of tissue can be processed per purification, depending on the type and RNA expression levels of the tissue. The system also incorporates a DNase treatment step to substantially reduce genomic DNA contamination, which can interfere with amplification-based methods. For best results from this system, use fresh samples when processing tissue. Older samples may yield less total RNA. If necessary freeze the samples immediately after collection in liquid nitrogen, and store at –70°C for future use. Samples homogenized in SV RNA Lysis Buffer also may be stored at –70°C.

For a protocol for RNA isolation from cultured cells, tissue samples, white blood cells, plant tissue, yeast and bacterial cells, see the *SV Total RNA Isolation System Technical Manual* #TM048.

Materials Required:

(see Composition of Solutions section)

- SV Total RNA Isolation System (Cat.# Z3100, Z3101 or Z3105) and protocol
- small tissue homogenizer (for RNA isolation from tissue)
- ethanol, 95%, RNase-free
- microcentrifuge
- 10X phosphate-buffered saline (PBS), sterile (for RNA isolation from cultured cells)
- sterile hypodermic syringe fitted with a sterile 20-gauge needle (for RNA isolation from cultured cells)
- water bath or heating block, preheated to 70°C
- Laboratory Vacuum Manifold (e.g., Vac-Man®, Cat.# A7231, or Vac-Man® Jr. Laboratory Vacuum Manifold, Cat.# A7660) and Vacuum Adapters (Cat.# A1331) (required for RNA purification by vacuum)

Poly(A)+ RNA Isolation Using the PolyATtract® System 1000

The PolyATtract® System 1000 isolates poly(A)+ RNA directly from crude cell or tissue lysates using the Promega MagneSphere® technology, eliminating the need for oligo(dT) cellulose columns. The system uses a biotinylated oligo(dT) primer to hybridize in solution to the 3′ poly(A) region present in most mature eukaryotic mRNA species. The hybrids are captured using streptavidin coupled to paramagnetic particles and a magnetic separation stand, then washed at high stringency. The mRNA is eluted from the solid phase by the simple addition of RNase-free deionized water. This procedure yields an essentially pure fraction of mature mRNA after only a single round of magnetic separation.

To isolate poly(A)+ RNA directly from tissue samples or cultured cells, see the *PolyATtract ® System 1000 Technical Manual* #TM228. This technical manual also describes precipitation and concentration of mRNA and determination of mRNA concentration.

Materials Required:

(see Composition of Solutions section)

- PolyATtract® System 1000 (Cat.# Z5400 or Z5420) and protocol
- small tissue homogenizer (for RNA isolation from tissue)
- 50ml sterile screw-cap conical tubes
- 15ml sterile COREX® or other glass centrifuge tubes
- 70°C water bath
- Beckman Model J2-21 centrifuge or equivalent
- 1X PBS (for RNA isolation from cell cultures)
- scale or balance (to weigh tissue samples)
- MagneSphere® Magnetic Separation Stand (see Table 1 of the *PolyATtract ® System 1000 Technical Manual* #TM228 to determine the appropriate magnetic stand)

B. DNA and RNA Labeling

A number of methods exist to attach a label to a nucleic acid molecule. These consist of techniques to incorporate the label into the substrate or attach the label to the ends of a nucleic acid fragment. The choice of method is determined largely by the nature of the substrate to be labeled. Some other factors to consider include: the amount of substrate available for labeling, its size in base pairs, the type of nucleic acid (DNA or RNA), the desired specific activity and whether it is double-stranded or single-stranded. Promega provides several nucleic acid-labeling systems, which are described briefly in this section.

Random-Primed Labeling

Random-primed labeling (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984) uses a mixture of random hexadeoxyribonucleotides to prime DNA synthesis in vitro from any linear double-stranded DNA template. With this method, it is possible to generate probes of high specific activity (>1 × 10^9cpm/µg), even using DNA fragments cut from agarose gels (Feinberg and Vogelstein, 1984). Since the input DNA serves as a template and remains intact during the reaction, minimal amounts of DNA (25ng) can be labeled to a high specific activity. Using the Prime-a-Gene® Labeling System, 40–80% of the labeled deoxyribonucleotide can typically be incorporated into the DNA template, depending on the template and reaction conditions used. Using a template greater than 500bp, probes generated by random-primed labeling generally are 250–300bp in length and are suitable for a variety of applications, including Northern analysis.

5′ End Labeling

5′ end labeling uses T4 polynucleotide kinase, which catalyzes the transfer of the γ-phosphate group from ATP to the 5′-hydroxyl terminus of double-stranded or single-stranded DNA or RNA molecules (the forward reaction). Suitable substrates include synthetic oligonucleotides, most of which lack a 5′-phosphate group, and DNA fragments that have been dephosphorylated with alkaline phosphatase to remove the 5′-phosphate groups. Under certain conditions, the reaction with T4 polynucleotide kinase can be made reversible, permitting exchange of the γ-phosphate of ATP with the 5′ terminal phosphate of a polynucleotide (the exchange reaction, see the *T4 Polynucleotide Kinase Technical Bulletin* #TB519), thus circumventing the need to dephosphorylate the substrate (Donis-Keller *et al*. 1977). The specific activity of a probe generated using the forward reaction is typically 2 × 10^6cpm/pmol, while the specific activity of a probe generated using the exchange reaction is approximately 6 × 10^5cpm/pmol (Berger and Kimmel, 1987). The Promega 5′ End-Labeling System includes both T4 Polynucleotide Kinase and Calf Intestinal Alkaline Phosphatase and their optimal reaction buffers to perform the dephosphorylation and labeling reactions (see the *DNA 5′ End-Labeling System Technical Bulletin* #TB096).

3′ End Labeling

Terminal deoxynucleotidyl transferase (TdT) is an enzyme that catalyzes the repetitive addition of mononucleotides from dNTPs to the terminal 3′-OH of a DNA initiator accompanied by the release of inorganic phosphate (Kato *et al*. 1967). The enzyme, which is available from Promega as Terminal Deoxynucleotidyl Transferase, Recombinant (Cat.# M1871), provides several methods to label the 3′ termini of DNA. The first involves adding an [α-^{32}P] dNTP "tail" to the 3′ termini of single-stranded DNA fragments. The number of nucleotides that will be added to the DNA template depends on the ratio of nucleotides to 3′-OH termini (Grosse and Manns, 1993). Alternatively, incorporation can be limited to a single nucleotide by using [α-^{32}P] cordycepin-5′-triphosphate, which lacks a free 3′ hydroxyl group, preventing incorporation of additional nucleotides (Tu and Cohen, 1980). The specific activity of probes generated by 3′ end labeling are typically 5 × 10^6cpm/µg (Brown, 1998).

Nick Translation

To label DNA by nick translation, free 3′-hydroxyl ends (nicks) are created within the unlabeled DNA by DNase I. DNA polymerase I then catalyzes the addition of a nucleotide residue to the 3′-hydroxyl terminus of the nick. At the same time, the 5′→3′ exonuclease activity of this enzyme removes the nucleotide from the 5′-phosphoryl terminus of the nick. The new nucleotide is incorporated at the position where the original nucleotide was excised, and the nick is thus shifted along one nucleotide at a time in a 3′ direction. This 3′ shift of the nick results in the sequential addition of labeled nucleotides to the DNA, while the pre-existing nucleotides are removed (Sambrook and Russell, 2001). DNA probes prepared by nick translation can be used for a wide variety of hybridization techniques, such as gel blots and colony plaque lifts. Typically greater than 65% of the labeled deoxyribonucleotide is incorporated, generating high-specific-activity probes (routinely 10^8dpm/µg) approximately 400–750 nucleotides in length (Sambrook and Russell, 2001).

in vitro Transcription

RNA probes can be synthesized by in vitro transcription (Melton *et al*. 1984) in the presence of a radioactive or non-radioactive label. Suitable radioactive labels include ^{32}P-, ^{33}P-, ^{35}S- or ^{3}H-labeled ribonucleotide. These probes have a defined length and are useful for Northern and Southern blots, in situ hybridization and RNase protection assays (Melton *et al*. 1984; Sambrook and Russell, 2001; Uhlig *et al*. 1991). Using an [α-^{32}P]rCTP label and the conditions described in the *Riboprobe® in vitro Transcription System Technical Manual* #TM016, RNA transcribed in vitro will typically have a specific activity of 2–2.5 × 10^8cpm/µg.

Additional Resources for DNA and RNA Labeling

Technical Bulletins and Manuals

TB049	*Prime-a-Gene® Labeling System Technical Bulletin*
9PIM187	*Terminal Deoxynucleotidyl Transferase, Recombinant, Promega Product Information*
TB096	*DNA 5′ End-Labeling System Technical Bulletin*
TB519	*T4 Polynucleotide Kinase Technical Bulletin*
TM016	*Riboprobe® in vitro Transcription Systems Technical Manual*

V. Composition of Solutions

Denhardt's Reagent, 50X (500ml)

5g Ficoll® (Type 400)
5g polyvinylpyrrolidone
5g bovine serum albumin (Fraction V)

Dissolve in DEPC-treated water, and adjust the volume to 500ml. Sterilize by filtration (0.45mm), and store at –20°C.

DEPC-treated water

Add diethyl pyrocarbonate (DEPC) to deionized water at a final concentration of 0.1%. Incubate overnight at room temperature in a fume hood. Autoclave for 20 minutes. **Caution:** DEPC is a suspected carcinogen. Work in a fume hood, and follow standard laboratory safety procedures.

MOPS 5X buffer (2L)

0.2M 3-[N-morpholino]-2-hydroxypropanesulfonic acid (MOPS) (pH 7.0)
0.05M sodium acetate
0.005M EDTA (pH 8.0)

To prepare 2 liters of buffer, add 83.72g of MOPS (free acid) and 8.23g of sodium acetate to 1.6 liters of DEPC-treated water, and stir until completely dissolved. Add 20ml of DEPC-treated 0.5M EDTA, and adjust the pH to 7.0 with 10N NaOH. Bring the final volume to 2 liters with DEPC-treated water. Dispense into 200ml aliquots, and autoclave. The solution will turn yellow, but this will not affect the quality of the buffer.

PBS (1L)

0.2g KCl
8.0g NaCl
0.2g KH_2PO_4
1.15g Na_2HPO_4

Add components one at a time to 900ml of room-temperature deionized water, and stir until completely dissolved. Adjust the pH to 7.4 using 1N HCl or 1N NaOH if necessary. Bring the final volume to 1 liter. If stored for long periods filter the solution through a 0.45mm filter, and store in a tightly capped sterile bottle.

Prehybridization/hybridization solution

50% deionized formamide
5X SSPE
2X Denhardt's Reagent
0.1% SDS

RNase digestion buffer

10mM Tris-HCl (pH 7.5)
5mM EDTA
200mM sodium acetate

RNA loading buffer

50% glycerol
1mMM EDTA
0.4% bromophenol blue

Prepare in nuclease-free water. Use very high-grade glycerol to avoid ribonuclease activity. Dispense into 500µl aliquots, and store at –20°C.

RNA sample buffer

10.0ml deionized formamide
3.5ml 37% formaldehyde
2.0ml MOPS 5X Buffer

Mix and dispense into 500µl aliquots. Store at –20°C in tightly sealed screw-cap tubes. These can be stored for up to 6 months. **Caution:** Formamide is a teratogen, and formaldehyde is a toxic carcinogen. Work in a fume hood, and follow standard laboratory safety procedures.

RPA hybridization buffer

80% deionized formamide
40mM PIPES (pH 6.4)
0.4M sodium acetate
1mM EDTA

RPA loading dye

80% deionized formamide
10mM EDTA
0.1% bromophenol blue
0.1% xylene cyanol
0.1% SDS

SSC, 20X (500ml)

87.7g NaCl
44.1g sodium citrate

Dissolve in 400ml of DEPC-treated water. Adjust the pH to 7.2 with 10N NaOH, and bring the volume to 500ml. Dispense into aliquots. Sterilize by autoclaving.

SSPE, 20X (1L)

175.3g NaCl
27.6g $NaH_2PO_4 \bullet H_2O$
7.4g EDTA, disodium salt

Dissolve in 800ml of DEPC-treated water. Adjust the pH to 7.4 with 10N NaOH, and bring the volume to 1 liter. Autoclave.

Stringency wash solution I

2X SSC
0.1% SDS

Stringency wash solution II

0.1X SSC
0.1% SDS

VI. References

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