2

Messenger RNA Accumulation

1. Concepts

The detection of mRNA levels of a particular gene is one of the cornerstones of molecular biology. There are many ways that mRNA can be detected, each with its strengths and weaknesses. Surprisingly, few investigators give much thought as to whether their methodologies and approaches are appropriate. Yet, the quantification and interpretation of results depends on understanding some key points. Three key factors contribute to the complexity and difficulty in examining differentially expressed genes. First, genes are not present at the same abundance and can vary from less than one up to thousands of copies per cell. This has implications for methods that must be used to accurately detect and quantify the expression of an mRNA. Second, the intensity of response varies greatly from gene to gene; that is, when comparing two treatments or conditions, an mRNA can be twofold or several orders of magnitude different between samples. Certain methods have a robust linear range and can handle both levels of response, while others are biased toward either the low or high responder. Last, there are many ways to alter gene expression, and some or all of the particular mechanisms may be at play. Approaches must be used that are capable of isolating, or at least accounting for, the competing possibilities so that hypotheses can be tested confidently. We will briefly discuss these parameters as they pertain to examining altered gene expression, and how these factors impinge on developing an optimal model system. Much of the following has been described in more detail in reference [14] as it pertained to cloning xenobiotically-induced genes.

1.1. mRNA Abundance

The mammalian genome of 3×10^9 base pairs (bp) has sufficient DNA to code for approx 300,000 genes, assuming a length of 10,000 bp per gene [15]. Obviously, not every gene is expressed by every cell; also, not every segment of DNA may be associated with a gene product. In fact, hybridization experiments in the mammalian cell have shown that approx 1 to 2% of the total sequences of nonrepetitive DNA are represented in mRNA [15]. Thus, if 70%

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of the total genome is nonrepetitive, 10,000–15,000 genes are expressed at a given time.

The average number of molecules of each mRNA per cell is called its representation or abundance. Of the 10,000 to 15,000 genes being expressed, the mass of RNA being produced per gene is highly variable. Usually, only a few sequences are providing a large proportion of the total mass of mRNA. Hybridization and kinetic experiments between excess mRNA and cDNA in solution identifies several components of mRNA complexity. Most of the mass of RNA (50%) is accounted for by a component with few mRNA species. In fact, approx 65% of the total mRNA may be accounted for in as few as ten mRNA species. The remaining 35% of the total RNA represents other genes being expressed in that tissue. Of course, the genes present in each category may be present in very different amounts and represent a continuum of expression levels. For means of this discussion, we will divide the three major components [16] into abundant, moderate, and scarce, representing approx 100,000 copies, 5000 copies and <10 copies per cell, respectively.

There are several reasons for discussing the components of mRNA. First, in a differential screen procedure (i.e., subtractive hybridization, differential display, microarray) what is actually being compared is two populations of mRNA, and you are examining the genes that overlap or form the intersection between these groups. When comparing two extremely divergent populations, such as liver and oviduct, as much as 75% of the sequences are the same [15] amounting to 10,000 genes that are identical and approx 3000 genes that are specific to the oviduct or liver. This suggests that there may be a common set of genes, representing required functions, that are expressed in all cell types. These are often referred to as housekeeping or constitutive genes. Second, there are overlaps between all com-

14. Vanden Heuvel, J.P. In: Toxicant-Receptor Interactions, Denison, M.S., Helferich, W.G., eds. Taylor and Francis, Philadelphia, PA. 1998, pp. 217–235

15. Lewin, B. Genes IV, Oxford University Press, Oxford UK.1990

16. Bishop, J. O. et al. Nature 270 (1974) 199–204.

15. Lewin, B. Genes IV, Oxford University Press, Oxford UK.1990

ponents of mRNA, regardless of the number of copies per cell. That is, differentially expressed genes may be abundant, moderate, or scarce. In fact, the scarce mRNA may overlap extensively from cell to cell, on the order of 90% for the liver-to-oviduct comparison. However, it worth noting that a small number of differentially expressed genes are required to denote a specialized function to that cell, and the level of expression does not always correlate with importance of the gene product.

As discussed in sections following, the key to developing an effective model for the study of differential gene expression may be to keep the differences in the abundant genes to a minimum. This is due to the fact that a small difference in expression of a housekeeping gene, say twofold, will result in a huge difference in the number of copies of that message from cell type to cell type (i.e., an increase of 10,000 copies per cell). Also, it is important to have a screening method that can detect differences in the scarce component. If the two populations to be compared have little difference in the abundant genes and you have optimized your screening technique to detect differences in the scarce population, the odds of cloning genes that are truly required for a specialized cellular function have increased dramatically.

1.2. Intensity of Response

A basic pharmacologic principle is that drugs and chemicals have different affinities for a receptor, and drug-receptor complexes will have different efficacies for producing a biological response, i.e., altering gene expression. A corollary of this principle states that not every gene being effected by the same drug-receptor complex will have identical dose-response curves. That is, when comparing two responsive genes, the affinity of the drug-receptor complex for the DNA response elements found in the two genes and the efficacy of the drug-receptor—DNA complex at effecting transcription could be quite different. In fact, similar DNA response elements may cause a repression or an induction of gene expression, depending on the context of the surrounding gene. Therefore, when comparing two populations of mRNAs (i.e., control versus treated), there may be dramatic differences in the levels of induction and repression regardless of the fact that all the genes are affected by the same drug-receptor complex.

Needless-to-say, the extent of change is important in the detection of these differences but not the importance of that deviation. For technical reasons, it is often difficult to detect small changes in gene expression (less than twofold). However, a twofold change in a gene product may have dramatic effects on the affected cell, especially if it encodes a protein with a very specialized or nonredundant function. Also, the detection of a difference between two cell popula-

tions is easier if the majority of the differences are in scarce mRNAs. Once again, this is owing to technical aspects of analyzing gene expression whereby the change from 500 to 1000 copies per cell is a dramatic effect compared to a change from 1×10^5 to 2×10^5, an effect that may be virtually unnoticed.

1.3. Specificity of Response

The last factor we will discuss regarding the complexity of mRNA species is that regulation of gene expression is multifaceted. The analysis of differential gene expression is most often performed by comparing steady-state levels of mRNA; that is, the amount of mRNA that accumulates in the cell is a function of the rate of formation (transcription) and removal (processing, stability, degradation). If differences in protein products are being compared, add translation efficiency and posttranslational processing to the scenario. With all the possible causes for altered gene expression, the specificity of response must be questioned. Is the difference in mRNA or protein observed an important effect on expression or is it secondary to a parameter in your model system you have not controlled or accounted for?

In the best-case situation, the key mechanism of gene regulation that results in the end point of interest should be known. At least, one should have criteria in mind for the type of response that is truly important. With most receptor systems, early transcriptional regulation may predominate as this key event. However, the other modes must be acknowledged, at least when novel genes are being characterized. By assuming that the key event is mRNA accumulation, the true initiating response such as protein phosphorylation or processing may be overlooked. Also, the extent and diversity of secondary events, i.e., those that require the initial changes in gene expression, may far exceed the primary events. The amplification of an initial signal (i.e., initial response [gene A] causes regulation of secondary response [gene B]) can confuse the interpretation of altered mRNA accumulation. Once again, one must have a clear understanding of whether a primary or secondary event is the key response and design your model accordingly.

2. Basic Methods and Approaches

For specific procedures on the analysis of gene expression the reader is directed to laboratory manuals such as Current Protocols in Molecular Biology [9] and Molecular Cloning [10]. Popular methods for examining mRNA accumulation are outlined in Fig. 2-1. As with any laboratory procedure, you must keep in mind the limitations of the procedure and your own technical expertise. In this section, the methods will be outlined broadly but with emphasis on the positive and negative aspects of each approach.

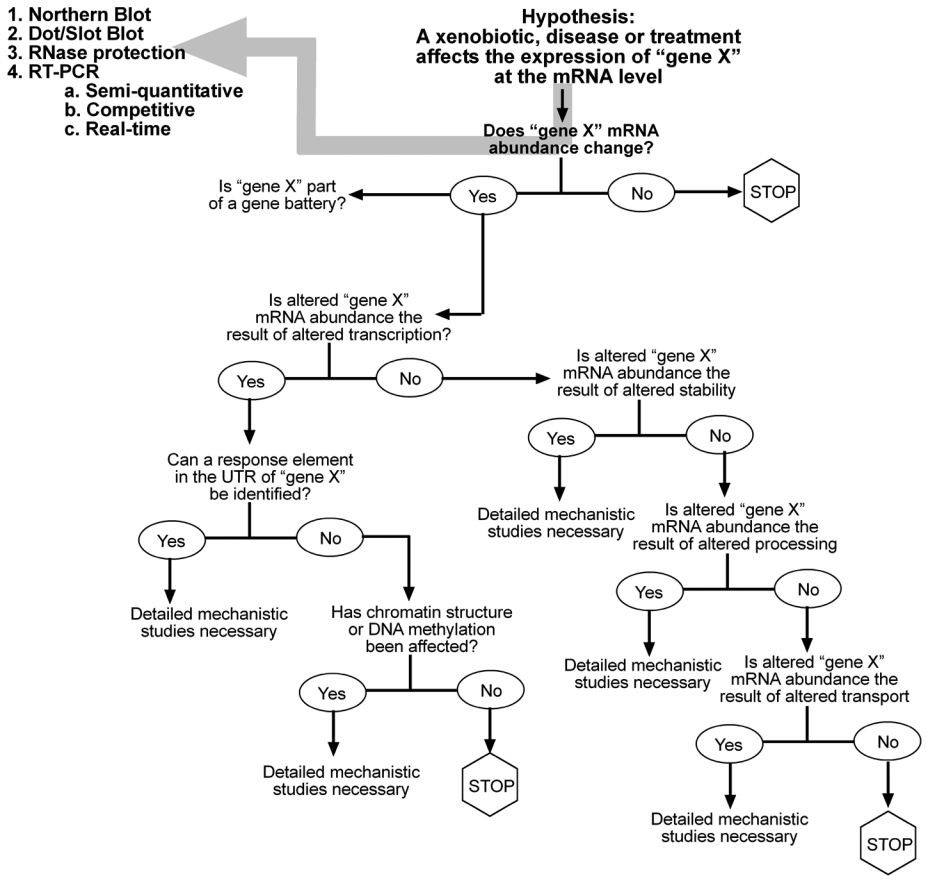

Fig. 2-1. Outline of approaches to examine mRNA accumulation.

Many are aware of the need for defining the model system when examining genome-wide mRNA accumulation, as when microarrays or serial analyses of gene expression (SAGE) are performed (Chapter 3). However, similar considerations should be made when using Northern blots or reverse transcriptase-polymerase chain reaction (RT-PCR) to examine a specific gene. Proper controls are necessary to minimize variability, and the studies must be designed in a way that eliminates extraneous factors (e.g., stress, contamination) that are not under investigation. In this section, approaches used to quantify the differences between two populations of mRNA for a particular gene will be examined. An assumption will be made that the model system is adequately designed to specifically test the hypothesis.

2.1. Northern Blots

An excellent resource for RNA analysis methods is www.ambion.com.

Despite the advent of more powerful techniques (RT-PCR, RNase protection), Northern blot analysis remains a standard method for detection and quantitation of mRNA levels. Northern blot analysis provides a relative comparison of message abundance between samples, is the preferred method for determining transcript size, and can be used for detecting alternatively spliced transcripts. The Northern blot procedure is straightforward and inexpensive, using common equipment and supplies present in most molecular biology laboratories.

The Northern blot measures mRNA levels. Some other "directional" assays include Southern (DNA), Western (protein) and Southwestern (protein–DNA) blots.

There are significant limitations associated with Northern blot analysis. First, despite the relative simplicity of the methodology, great pains must be taken to avoid RNase contamination of solutions and plasticware. If RNA samples are even slightly degraded, the quality of the data and the ability to quantitate expression are severely compromised. Second, Northern blotting is much less sensitive than nuclease protection assays and RT-PCR. Rare genes require highly specific probes and large amounts of RNA. Enriching the sample for polyadenylated mRNA and optimizing hybridization conditions can improve sensitivity to some extent. Third, Northern blots are not amenable to high-throughput analysis. For example, although it is possible to detect more than one gene per blot, often this requires stripping the nylon membrane and reprobing,which is time consuming and problematic, since harsh treatment is required to strip conventional probes from blots. Last, this procedure is most often used as a relative quantitation method, since the gene in question is being examined as a function of the expression of a housekeeping gene. Although internal standards can be synthesized and spiked into the sample (e.g., a synthetic gene with the hybridization site of the probe) in order to generate a standard curve and to obtain absolute quantification, this is rarely performed. Thus Northern blotting requires a large difference between samples (five- to tenfold) to be significant.

The general approach of Northern blot analysis is as follows (*see* Fig. 2-2). Extraction of high-quality intact RNA is a critical step in performing Northern analysis. This is generally performed by cell lysis with detergents or solvent, inhibition of ribonuclease, and ultimately separation of proteins and DNA from the RNA. This later event can be performed by liquid phase separation or via oligo(dT) chromatography. Once RNA samples are isolated, denaturing agarose gel electrophoresis is performed. Formaldehyde has traditionally been used as the denaturant, although the glyoxal system has several advantages over formaldehyde. All buffers and apparatus must be painstakingly treated with RNase inhibitors. Following separation by denaturing agarose gel electrophoresis, the RNA is transferred to a positively charged nylon membrane and then immobilized for subsequent hybridization. The transfer may be performed using a passive, slightly alkaline elution or via commercially available active transfer methods (electroblotter, pressure blotter). The membrane is crosslinked by ultraviolet light or by baking. Northern blots can be probed with radioactively or nonisotopically labeled RNA, DNA, or oligodeoxynucleotide probes. DNA probes may originate from plasmids or PCR. RNA probes can be produced by in vitro transcription reactions. Radioactivity may be incorporated during the PCR or in vitro transcription reactions or may be performed via end-labeling or random priming. Prehybridization, or blocking, is required prior to probe hybridization to prevent the probe from coating the membrane. Good blocking is necessary to minimize background problems. Although double-stranded DNA probes must be denatured prior to use, RNA probes and single-stranded DNA probes can be diluted and then added to the prehybridized blot. After hybridization, unhybridized probe is removed by washing in several changes of buffer. Low-stringency washes (e.g., with 2X SSC) remove the hybridization solution and unhybridized probe. High-stringency washes (e.g., with 0.1X SSC)

There are many commercially available kits for the extraction of total RNA (mRNA, tRNA, rRNA) or poly(A) RNA (mRNA). Most methods for total RNA utilize chloroform:phenol extraction, whereas mRNA can be enriched via oligo(dT) chromatography.

SSC buffers are commonly employed in nucleic acid hybridizations. 20X SSC consists of 3 M NaCl, 0.3 M Na3 citrate.

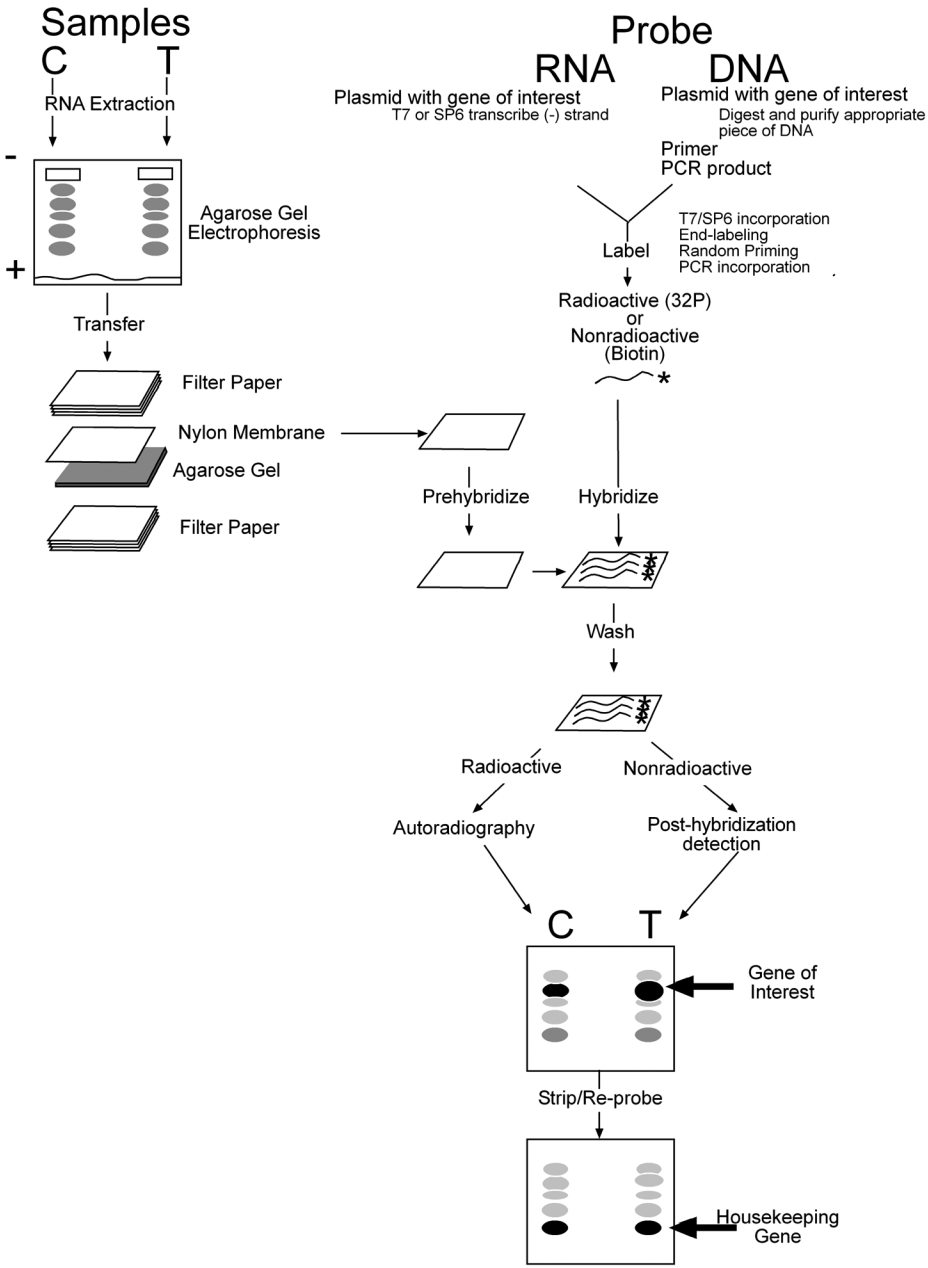

Fig. 2-2. Basic procedures in Northern blotting.

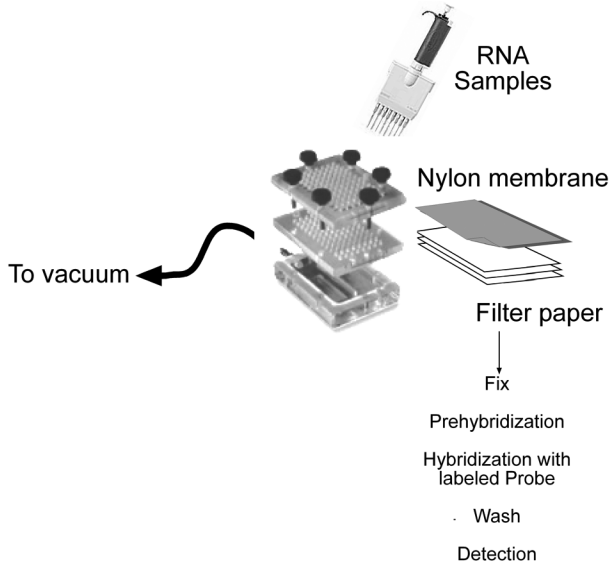

Fig. 2-3. Dot- or slot-bot analysis of RNA.

remove partially hybridized molecules. If a radiolabeled probe was used, the blot can be wrapped in plastic wrap to keep it from drying and then immediately exposed to film for autoradiography. If a nonisotopic probe was used, the blot must be treated with nonisotopic detection reagents prior to film exposure. Standard methods for removing probes from blots to allow subsequent hybridization with a different probe often include harsh treatments with boiling 0.1% SDS or autoclaving.

2.2. Dot/Slot Blots

Dot or slot blot analysis takes its name from the apparatus used to apply the sample to the nylon membrane, as shown in Fig. 2-3. This method is analogous to the Northern blot in most ways but has a much higher throughput, as dozens of samples can be examined simultaneously. RNA samples are applied to the membrane and fixed by UV or heat crosslinking. The subsequent application of probe, washing and detection is as described for Northern blots. The main advantage of the dot/slot blot is the number of samples that can be run simultaneously and the relative ease of quantitation resulting from uniformity in bands to be analyzed. There may be some increased sensitivity in the assay because more sample can be loaded onto the matrix, as long as the nitrocellulose or nylon does not become saturated. In addition, the RNA can be of slightly lower quality and still give a detectable signal. However, sensitivity is still signifi-

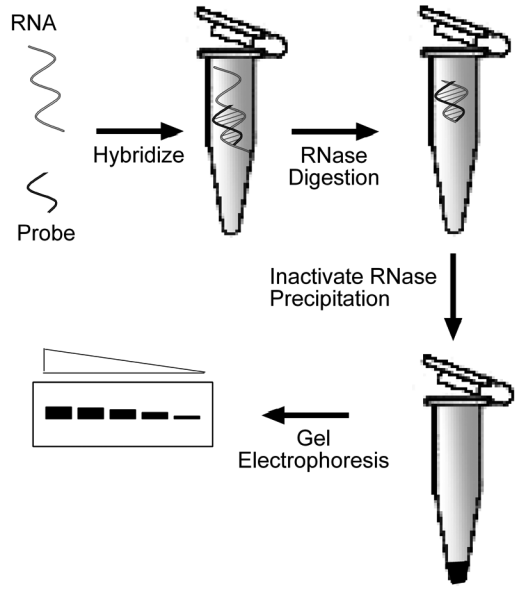

Fig. 2-4. Basics of RNase protection assays (RPAs).

cantly less than that for ribonuclease protection assay or RT-PCR. The dot/slot blot does not result in any information about transcript size, and conditions must be optimized to ensure that the probes are specific for the target gene.

It is also possible to perform a "reverse Northern" using the dot/slot blot format. In this procedure, the probe is spotted onto the nylon and the RNA sample is labeled (isotopic or nonisotopic) and added to the hybrizidation solution. This allows for the examination of multiple genes in one sample. The reverse Northern is predecessor to the microarray assay that will be discussed in detail in Chapter 3.

2.3. RNase Protection Assays (RPA)

Ribonuclease (or RNase) protection assays (RPAs) are an extremely sensitive method for the detection and quantitation of specific RNAs in a complex mixture of total cellular RNA. RPA is a solution hybridization of a single-stranded antisense probe to an RNA sample (*see* Fig. 2-4). The small volume solution hybridization is far more efficient than more common membrane-based hybridization and can accommodate much more RNA (hence increasing sensitivity over Northern blotting). After hybridization, any remaining unhybridized probe and sample RNA are removed by digestion with a mixture of nucleases (usually RNase A and T1; *see* Table 2-1). The nucleases are inacti-

vated, usually by extraction with chloroform/phenol, and the remaining RNA-probe hybrids are precipitated. These products are separated on a denaturing polyacrylamide gel and are visualized by autoradiography or secondary detection. To quantitate mRNA levels using RPAs, the intensities of probe fragments protected by the sample RNA are compared to the intensities generated from either an endogenous internal control (relative quantitation) or known amounts of sense strand RNA (absolute quantitation).

As just mentioned, one major advantage of RPAs over membrane-constrained hybridization is sensitivity. Low-abundance genes are detectable in RPAs, or a small sample size may be used for moderate or high expressing species. In addition, RPAs are the method of choice for the simultaneous detection of several RNA species. During solution hybridization and subsequent analysis, individual probe-target interactions are for all intents and purposes independent of one another. Thus, several RNA targets and housekeeping genes can be assayed simultaneously if the protected fragments are of different lengths. In addition to their use in quantitation of RNA, various RNases can be used to map the structure of transcripts, as will be discussed in more detail in Chapter 4 and 5.

The basic steps of the typical RNase protection assay are relatively straightforward and utilize common materials found in molecular biology laboratories (Fig. 2-4). RNA is extracted as described for the Northern blot analysis. RPA requires RNA probes, most often prepared using in vitro transcription assays and either radiolabeled or nonisotopically labeled. Oligonucleotides and other single-stranded DNA probes can only be used in assays containing S1 nuclease. Optimization of an RPA assay is very probe- and gene-specific and depends mostly on transcript concentration. Probe concentration must be in molar excess over the target mRNA to ensure rapid hybridization. Thus, highly abundant targets

Table 2-1
RNase Properties

RNase	Specificity
RPA	
A	3' of ss C and U
I	3' of ssNTP
T1	3' of ss G
S1 nuclease	ssRNA
RNA structure	
H1	RNA of RNA/DNA
V1	dsRNA

ss, single stranded; ds, double stranded

Ambion (www.ambion.com) suggests the following dilutions of labeled to unlabeled probe:

- *Low abundance, 1:0*
- *Moderate abundance,1:50*
- *Abundant, 1:10,000*

require the use of low specific-activity probes for abundant targets (dilute labeled probe with unlabeled) whereas probes for scarce RNA targets require no dilution. Generally, 10 µg total RNA is hybridized overnight with approx 50,000 cpm for each probe.

Comparison of S1 nuclease and RNase A/T1 RPA can be found at: http:/www.promega.com/pnotes/38/38_01/38_01.htm. Accessed 03/24/06.

Although S1 nuclease is able to cleave after every residue in both RNA and single-stranded DNA, some problems are associated with its use in RPA assays. Lower incubation temperatures and high salt concentrations (>200 m*M*) must be used to favor single-stranded over double-stranded cleavage, and S1 nuclease is prone to nonspecific cleavage in AU-rich regions. Thus, mixtures of RNase A and T1 typically have been used for digestion of unhybridized RNA in solution hybridization experiments. Nuclease P1 has been substituted for RNase A when using AU-rich probes which require lower reaction temperatures. The amount of enzyme(s), buffer constituents and incubation conditions vary widely from protocol to protocol and may require some optimization. Following digestion, the RNases need to be inactivated. Phenol/ chloroform extraction is required to inactivate Nuclease P1 and RNases A and T1. This extraction is not required for some of the commercially available RNase mixtures and addition of ethylenediaminetetraacetic acid (EDTA) and ethanol precipitation is sufficient.

The percentage of polyacrylamide in gel electrophoresis depends on the size of the products to be examined. Typically a 6% denaturing acrylamide gel is used to resolve fragments of 300–1000 nucleotides. The detection and analysis of the band intensities depends on whether radioactivity or nonisotopic methods were used.

2.4. RT-PCR

Polymerase chain reaction is an enzymatic assay which is capable of producing large amounts of a specific DNA fragment from a small amount of a complex mixture (Fig. 2-5; reviewed in [17]). In RT-PCR, the mRNA must first be converted to a

17. Mattes, W. B. In: PCR Protocols in Molecular Toxicology, Vanden Heuvel, J.P., ed. CRC Press, Boca Raton, FL. 1997, pp. 1–40.

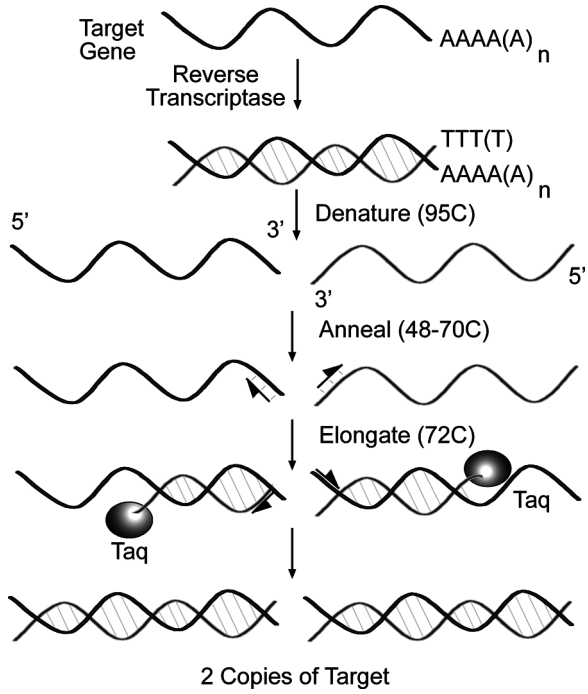

Fig. 2.5. Reverse transcription and one cycle of PCR.

double-stranded molecule by using the enzyme reverse transcriptase (RT). The thermostable DNA polymerase (i.e., Taq) and the use of specific "primers" are the key features of any PCR reaction. All known DNA polymerases require deoxyribonucleotide triphosphates (dNTPs), a divalent cation (Mg^{2+} or Mn^{2+}), a DNA or cDNA template, and a region of that template that is double stranded adjacent to a single-stranded nick or gap. The double-stranded region is provided by the primer annealing to its complementary region of the DNA template. If the starting mixture includes not only a single-stranded polynucleotide template, but also (1) its complementary strand and (2) two oligonucleotide primers that hybridize to both strands, copies of both of these strands will be produced each cycle, and these copies can be used as templates for subsequent cycles. Short DNA fragments whose ends are defined by the position of the two oligonucleotide primers will accumulate in an exponential fashion, i.e., like a chain reaction. If 30 cycles of PCR are performed, theoretically one will achieve a 2^{30} amplification of the target gene's cDNA. The product which is formed is specific for a particular transcript as dictated by the design of the oligonucleotide primers.

Common RT-PCR Definitions

Internal standard (IS): A type of control molecule that can be used to minimize tube-to-tube variability in amplification efficiency. Normally an IS is a synthetic molecule that contains the same recognition sequences as the gene of interest. This type of amplification control is spiked into the PCR reaction.

External standard (ES): A type of control that can be used to minimize differences in template (mRNA, cDNA, or DNA) concentration from sample to sample. Most often an ES is a housekeeping gene that is used in a "coamplification" type of quantitation. This control is not added to each sample, as it is present in a finite amount in each tube. Care must be taken to ensure that the housekeeping gene is not affected by the treatment condition. Typical external standards include actin, tubulin, 18S rRNA, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Template: Any cDNA or DNA that contains primer recognition sites and can be PCR amplified.

Target gene: The gene of interest; to be differentiated from the internal standard, external standard, or artifact templates.

Linker gene: Specifically used to describe a template used to create a synthetic molecule in the synthesis of an internal standard

Forward primer (FP): Analogous to the 5' or "upstream" primer (usp).

Reverse primer (RP): Same as the 3' or "downstream" primer (dsp).

Cross-over point: In competitive RT-PCR, the concentration of internal standard at which the PCR products for the target and the internal standard are equivalent.

Therefore, RT-PCR is a tool to examine the messenger RNA expression of a target gene in that the amount of product formed is a function of the amount of starting template. Of course, the examination of mRNA accumulation can be determined in many cases by hybridization procedures such as Northern blots, dot or slot blots, and RNase protection assays. Nonetheless, in terms of amount of sample required, detection of small differences in expression and ability to examine many genes in a large number of samples, RT-PCR stands above the more conventional procedures.

2.4.1. Competitive RT-PCR

The predominant negative of RT-PCR is related to its ability to amplify the products. That is, an internal standard (IS) is necessitated in these assays owing to the fact that there is a large amount of tube-to-tube variability in amplification efficiency. For example, if ten tubes of seemingly identical reagents are PCR amplified, there could be as much as a threefold difference in the amount of product formed. If an IS was coamplified with the target, the efficiency of amplification in each tube could be corrected and this threefold difference could easily be negated.

A good internal standard for quantifying mRNA levels depends on many factors. The reader is directed to other sources [18] for a description of IS construction methods and considerations. In addition, once an IS is produced or obtained, the investigator must select from several methods for the actual RT-PCR assay. However, in general, the "competitive" RT-PCR approach is the easiest and most adaptable procedure. The standard competitive RT-PCR approach is shown in Fig. 2-6. The basis for this method is that the greater the quantity of competitor (i.e., IS) that is present, the less likely it is that the primers and Taq DNA polymerase will bind to the target cDNA and amplify it. Therefore, despite the fact that all reagents are present in excess (i.e., primers, enzyme, $MgCl_2$, nucleotides) the reaction appears to be competitive. As the amount of competitor is increased, less and less target PCR product is formed until eventually only the IS product is observed. When a dilution series of IS is spiked into a constant amount of RNA, it is possible to estimate the amount of a specific product present in the sample. As depicted in Fig. 2-6, an increase or decrease in target mRNA is easy to visualize using competitive RT-PCR. In addition, calculation of the amount of transcript mRNA in each sample is quite straightforward if one has access to standard laboratory photographic and densitometric equipment [18]. In general terms, the amount of IS required to result in a 1:1 ratio of IS to target PCR product is representative of the amount of transcript present in the original sample; for example, in Fig. 2-6, 10^6 molecules target mRNA in total RNA derived from control versus 10^7 from treated cells. As small as a twofold difference between exposure groups can be routinely identified using a very small amount of RNA (i.e., 10–100 ng total RNA, the equivalent of approximately 1000–10,000 cells).

18. Vanden Heuvel, J.P. In: PCR Protocols in Molecular Toxicology, Vanden Heuvel, J.P., ed. CRC Press, Boca Raton, FL. 1997, pp. 41–98.

2.4.2. Relative RT-PCR

Relative RT-PCR uses primers for an external control that are coamplified ("multiplexed") in the same RT-PCR reaction with the gene-specific

18. Vanden Heuvel, J.P. In: PCR Protocols in Molecular Toxicology, Vanden Heuvel, J.P., ed. CRC Press, Boca Raton, FL. 1997, pp. 41–98.

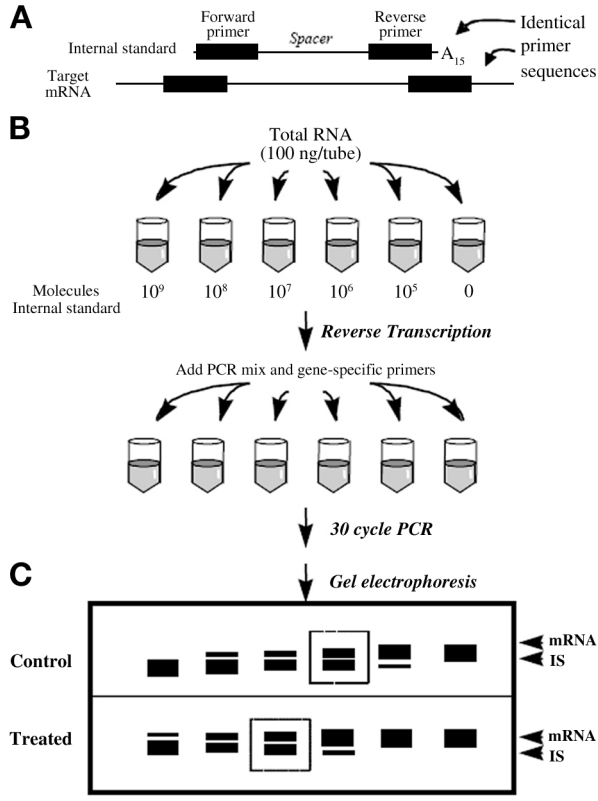

Fig. 2-6. Basic competitive RT-PCR. An internal standard (IS) is constructed, as depicted in **(A)**, that contains the same primers as the target gene but whose resultant product is of different length. In basic competitive RT-PCR, a constant amount of RNA is used and a dilution series of IS is spiked into each tube (10^9–10^5 molecules/tube) as shown in **(B)**. A representative ethidium bromide-stained agarose gel is shown in **(C)**. Note that as the amount of IS is increased the intensity of the target product decreases. Also, the equivalency, or crossover, point is higher in the treated samples than control, indicative of induction of target mRNA. (Adapted from [19].)

An optimal internal standard for competitive RT-PCR should amplify with equal efficiency as the target transcript. This can be achieved by designing the competitor with the same primer recognition sequence as the target but with slightly different product size.

19. Gilliland, G.S. et al. In: PCR Protocols: A Guide to Methods and Applications, Innis, M.A., ed. Academic, San Diego, CA. 1990, pp. 60–69.

primers. Common internal controls include β-actin, tubulin, and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNAs and 18S rRNA. This method is quite straightforward but requires more

optimization than one would anticipate. External control and target gene primers must be compatible and not produce additional bands or hybridize to each other. Also, salt concentration, annealing temperature, and pH must be controlled to assure that both products are amplifying efficiently. Quantitation must be performed during the nonplateau phase of the amplification for both the target and external control; often, this affects the sensitivity of the assay since samples are examined at a relatively low number of cycles (20–25 cycles). If the efficiency of the primer pairs are quite dissimilar, it may be necessary to dilute the primers for the more robust reaction. The expression of the external control should be constant across all samples being analyzed and this signal can be used to normalize sample data to account for tube-to-tube differences caused by variable RNA quality or RT efficiency, inaccurate quantitation, or pipetting.

Real-time RT-PCR has become the method of choice for quantifying mRNA accumulation due to its high-throughput capabilities

2.4.3. Real-Time RT-PCR

Real-time RT-PCR combines the best attributes of both relative and competitive RT-PCR in that it is accurate, precise, high throughput, and relatively easy to perform. Real-time PCR gets its name from the fact that reaction products are quantitated for each sample in every cycle. The result is a large (10^7-fold) dynamic range, with high sensitivity and speed. Preoptimized kits for thousands of genes are available and they greatly reduce the time required to generate data. However, the procedure requires expensive equipment and reagents, which may be prohibitive to some investigators.

Preoptimized kits for real-time PCR quantitation can be obtained from Applied Biosystems Inc. (ABI) at http://appliedbiosystems.com. Accessed 03/24/06.

Real-time PCR systems rely on the detection and quantitation of a fluorescent reporter. Since the products are analyzed in real time, there is no need for post-PCR manipulation. This decreases the chance for experiment-to-experiment contamination and eliminates the variability inherent in agarose gel electrophoresis and quantitation. Currently, there are two major methods that are used in real-time

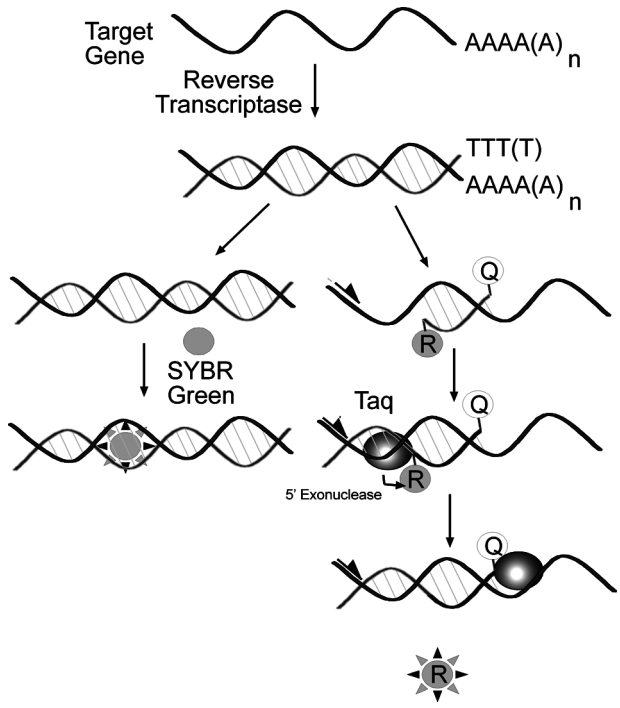

Fig. 2-7. Real-time PCR.

PCR: DNA fluorescent dyes (SYBR green) and fluorescent resonance energy transfer (FRET; i.e., TaqMan(r), Molecular Beacons). The amount of fluorescence in each case increases as the amount of PCR product increases. *See* Fig. 2-7 for a summary of the two methods.

Of the DNA fluorescent dyes, SYBR green is the most common. SYBR green is an intercalating agent that binds double-stranded DNA, and in this bound form upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases. The advantages of SYBR green are that it's inexpensive, easy to use, and sensitive. The disadvantage is that SYBR green will bind to any double-stranded DNA in the reaction, including primer-dimers and other nonspecific reaction products, which results in an overestimation of the target concentration.

The TaqMan and Molecular Beacons approaches both utilize fluorescent probes and FRET for quantitation. This assay utilizes the 5' nuclease activity of Taq polymerase. TaqMan probes are oligonucleotides that contain a fluorescent dye on the 5' end (i.e., carboxyfluorescein [FAM]), and a quenching dye on the 3' base (i.e., tetramethylrhodamine [TAMRA]). These probes are designed to hybridize between the standard PCR primers. Prior to enzymatic activity, the

excited fluorescent dye (FAM) transfers energy to the nearby quenching dye molecule (TAMRA), which has a much weaker emission, resulting in a nonfluorescent probe. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5' exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent and quenching dyes and FRET no longer occurs. Fluorescence increases in each cycle, proportional to the rate of probe cleavage.

Probes used as molecular beacons are utilized in a similar manner to TaqMan, but exonuclease activity is not required. Molecular beacons also contain fluorescent and quenching dyes and utilize FRET, but the two dyes are incorporated within the probe and are not accessible to Taq's exonuclease function. Molecular beacons are designed to adopt a hairpin structure while free in solution, bringing the fluorescent dye and quencher in close proximity. Upon hybrization to the target gene, the fluorescent dye and quencher are separated, interrupting the FRET and enhancing the signal from the probe. Unlike TaqMan probes, molecular beacons are designed to remain intact during the amplification reaction, and must rebind to target in every cycle for signal measurement.

3. Summary

The examination of transcript concentrations is one of the fundamental experiments in modern biology. The methods currently in use for the quantification of mRNA accumulation are summarized in Table 2-2, with the identifiable strengths and weaknesses of each. Perhaps the two most common methods are Northern blots and RT-PCR. Northern blots are insensitive and relatively nonquantitative. However, they allow for visualization of transcript size, utilize well-established, straightforward techniques, and are inexpensive to perform. In contrast, RT-PCR (quantitative, real-time) are exquisitely sensitive and robust, with the capability for high-throughput gene expression analysis. RT-PCR requires expensive equipment and must be performed with care to minimize contamination and tube-to-tube variability. Realtime PCR has become the method of choice for high-throughput gene expression analysis.

Table 2-2
Summary of Pros and Cons of Methods to Examine mRNA Accumulation

Method	Pros	Cons	Quantitation
Northern blot	• Technically simple and inexpensive • No amplification or RNA manipulation • Can gain information on transcript size or splicing	• Insensitive, requiring large sample size • Low throughput • Sensitive to RNA quality	• Generally requires large differences in expression between two samples • Concentration is often expressed relative to a housekeeping gene • An exogenous standard may be spiked for absolute quantitation
Dot/Slot blot	• Technically simple and inexpensive • No amplification or RNA manipulation • High throughput	• Relatively insensitive • No information on transcript size or quality	• Concentration is often expressed relative to a housekeeping gene • Standard curves may be generated for absolute quantitation
RNase protection	• Increased sensitivity • Less sensitive to partial RNA degradation • Several genes can be examined concurrently	• No information on transcript size or quality • Difficult to optimize	• Concentration is often expressed relative to a housekeeping gene • Standard curves may be generated for absolute quantitation
RT-PCR	• Most sensitive and quantitative method • High throughput and rapid	• Technically challenging • Potential for contamination (DNA in sample; experiment to experiment; tube to tube) • Competitive PCR requires synthesis of internal standard • Requires expensive equipment (in particular, real-time PCR)	• Relative PCR requires amplification in exponential phase; coamplification performed with housekeeping gene. Generally requires large differences between samples • Competitive PCR uses a synthetic molecule with primer sites. Standard curves are prepared. Small differences in expression can be quantified. • Real-time PCR detects the number of transcripts produced during amplifica-- tion. Sensitive and high throughput with small differences between samples detected.

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