Part I
Analytical Methods

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RNA Methodologies

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Keywords

Chaotropic
Biologically disruptive. Chaotropic lysis buffers disrupt the cell and organelle membranes and destroy enzymatic activity on contact.

Complementary DNA (cDNA)
DNA synthesized *in vitro* from an RNA template by an enzyme known as a reverse transcriptase. cDNA can be either single- or double-stranded, and is used for RT-PCR, nucleic acid probe synthesis, or library construction. Because cDNA can only be made from transcripts present at the moment of cellular disruption, it is a permanent biochemical record of the cell.

Dot-blot
A membrane-based technique for the quantification of specific RNA or DNA sequences in a sample. The sample is usually ‘‘dot’’-configured onto a filter by vacuum filtration through a manifold. Dot blots lack the qualitative component associated with electrophoretic assays.

Functional genomics
Response of the genome, such as changes in gene expression, as a consequence of experimental challenge. This most often involves the up- and downregulation of specific genes.

Heterogeneous nuclear RNA (hnRNA)
The primary product of RNA polymerase II transcription in eukaryotic cells. hnRNA alone is processed and matured into mRNA which, in turn, is able to support the synthesis of proteins, though some hnRNA molecules fail to mature and are degraded in the nucleus.

Housekeeping gene
A gene that is expressed, at least theoretically, at a constant level in the cell. The products of these genes are generally required to maintain cellular viability or normal function. Housekeeping genes are often assayed as purportedly invariant controls, compared to the modulation of other genes in response to experimental challenge. Almost all known housekeeping genes show varying levels of gene expression under specific circumstances, so there is no single all-purpose housekeeping gene.

Hybridization
The formation of hydrogen bonds between two complementary nucleic acid molecules. The specificity of hybridization is a direct function of the stringency of the system in which the hybridization is being conducted.

Messenger RNA (mRNA)
The mature product of RNA polymerase II transcription. mRNA is derived from heterogeneous nuclear RNA (hnRNA) and, in conjunction with the protein translation apparatus, is capable of directing the synthesis of the encoded polypeptide.

Noncoding RNA (ncRNA)
A diverse population of transcripts in the cell that do not encode proteins or polypeptides. Certain classes of noncoding RNAs have been shown to profoundly regulate the expression of other genes.

Northern blot analysis
A technique for transferring RNA from an agarose gel matrix, after electrophoresis, onto a filter paper for subsequent immobilization and hybridization. The information gained from Northern blot analysis is used to assess, both qualitatively and quantitatively, the expression of specific genes, though much more sensitive methods are available.

Nuclear runoff assay
A method for labeling nascent RNA molecules in isolated nuclei. The rate at which specific RNAs are being transcribed can then be assayed based upon the degree of label incorporation. See "Steady-state RNA" for comparison.

Nuclease protection assay
A method for mapping and/or quantifying RNA transcripts. In general, hybridization between probe and target RNA takes place in solution, followed by nuclease digestion (with S1 nuclease or RNase) of all molecules or parts thereof which do not actually participate in duplex formation. Nucleic acid molecules which are locked up in a double-stranded configuration are relatively safe or protected from nuclease degradation. The undigested RNA:RNA or RNA:DNA hybrids are then precipitated and/or electrophoresed for quantification.

Poly(A)$^{+}$ tail
A tract of up to 250 adenosine residues enzymatically added to the 3′ terminus of mRNA by the nuclear enzyme poly(A) polymerase. The addition of a poly(A) tail involves cleavage of the primary transcript, followed by polyadenylation. Most (but not all) eukaryotic mRNAs exhibit this structure which stabilizes their 3′ terminus. The poly(A) tract is commonly targeted by oligo(dT) for selection of these transcripts, as well as for priming the synthesis of first-strand cDNA.

Polymerase chain reaction (PCR)
Primer-mediated, enzymatic amplification of specific cDNA or genomic DNA sequences. This technology revolutionized molecular biology in the early and mid-1990s; it is the best known and perhaps most widely used molecular biology technique.

Primer
An artificially synthesized, short single-stranded nucleic acid molecule that can base-pair with a complementary sequence and which provides a free 3′-OH for any of a variety of primer extension-related reactions, especially PCR.

Probe
A DNA or RNA molecule which carries a label allowing it to be localized and quantified throughout an experiment. Probes are used most often to hybridize to complementary sequences present among a plethora of different molecules in a nucleic acid sample, as in Northern analysis, Southern analysis, nuclease protection analyses, or DNA library screening.

Proteome
The full complement of proteins produced by a cell at a particular time. Proteome maps are typically generated and assessed by two-dimensional electrophoresis and

other techniques designed to identify, quantify, and characterize the products of translation.

Real-time PCR
A state-of-the art method for measuring PCR product accumulation as it is produced in each cycle, rather than measuring the final product mass at the end of the reaction (end-point PCR). Real-time PCR is widely regarded as the premier quantitative molecular biology technique and, as such, is often referred to as quantitative PCR (qPCR).

Relative abundance
The quantity of a particular RNA transcript relative to some other transcript in the same sample, or relative to the amount of the same transcript in other experimentally related samples. This determination is most often made using PCR-based analysis, though less quantitative, non-PCR assays may also be used.

Ribonuclease (RNase)
A family of resilient enzymes which rapidly degrade RNA molecules. The control of ribonuclease activity is a key consideration in all manipulations involving RNA.

Ribonucleic acid (RNA)
A polymer of ribonucleoside monophosphates, synthesized by an RNA polymerase. RNA is the product of transcription.

RNA interference (RNAi)
A novel method by which specific mRNA transcripts can be transiently prevented from participating in translation, or which are destroyed altogether through the formation of a dsRNA molecule. RNAi is ''loss-of-function'' approach used to determine the role of a specific gene; it is also known as post-transcriptional gene silencing.

Reverse transcription PCR (RT-PCR)
The technology by which RNA molecules are converted into their complementary DNA (cDNA) sequences by any one of several reverse transcriptases, followed by the amplification of the newly synthesized cDNA using PCR. Not to be confused with real-time PCR, which may or may not involve the use of RNA.

Steady-state RNA
The final accumulation of RNA in the cell. For example, measurement of the prevalence of a particular species of mRNA in a sample does not necessarily correlate with the *rate* of transcription or RNA degradation in the cell (see Nuclear runoff assay).

Transcription
The process by which RNA molecules are synthesized from a DNA template.

Transcriptome
The complete set of RNA molecules produced by a particular cell under a particular set of circumstances.

Cellular biochemistry is reflected in the abundance of cellular RNA species which, inevitably, drives the phenotype of the cell. In order to understand more readily the cellular response to experimental or environmental challenges, various subpopulations of RNA are harvested and characterized to gain insight to differential expression of genes, and possibly also the subcellular level at which these genes are modulated. RNA is isolated to answer transcription questions by measuring the prevalence of one or more RNA species. The observed changes in transcript abundance may then be related to morphological or physiological differences in the cells or tissues under investigation. The expedient isolation of high-quality RNA is essential to support all downstream applications, and the methods to be used are dictated by the nature of the biological source material. The RNA methodologies are diverse, with each providing a glimpse of some aspect of gene regulation with a characteristic level of sensitivity. Each technique has both advantages and limitations, often requiring a combination of RNA-based assays to provide a more complete picture of the upregulation and downregulation of specific genes and gene families. Data from transcription-based assays are often complemented by quantifying the cognate protein(s), the levels of which often – but not always – correlate. Most investigators use RNA, rather than protein, as a parameter of gene expression because RNA is often easier to isolate than proteins, and because very rare transcripts can be detected via cDNA synthesis and PCR amplification. Presently, there is no such powerful amplification method for proteins.

1 Introduction

The isolation and characterization of ribonucleic acid (RNA) from cells and tissue samples is a central and recurrent theme in molecular biology. In particular, the purification of chemically stable and biologically functional RNA is the starting point for the systematic evaluation of cellular biochemistry by standard molecular methods, including all forms of reverse transcription polymerase chain reaction (RT-PCR), as well as time-honored methods such as Northern analysis, nuclease protection (S1 and ribonuclease (RNase) protection assays), nuclear runoff assay, complementary DNA (cDNA) library construction, and even dot-blot analysis. Messenger RNA (mRNA) abundance is a useful parameter of gene expression; therefore, the expedient recovery of RNA from a biological source is a critical first step for the derivation of meaningful data. Difficulties in the purification, handling, and storage of RNA are intrinsic to the labile chemical nature of these molecules. These difficulties are further compounded by the aggressive character of resilient RNases, the apparent ubiquity of which is undisputed. Indeed, the novice quickly learns of the absolute requirement for management of RNase

activity at each level of RNA isolation and characterization. Failure to do so will almost certainly compromise the integrity of the resulting RNA and its probable utility in various downstream applications.

2 Subpopulations of RNA

Prior to the onset of cellular disruption, the investigator must determine *which* RNA subpopulation is of experimental interest. For example, the precise questions being asked of a particular set of experiments may require characterization of the total cellular RNA, the cytoplasmic RNA alone, nuclear RNA alone, poly(A)$^+$ RNA, or even noncoding RNA species. Transcriptional activity is generally assayed using one of the methods described below, such as Northern analysis, and the data are then validated using another method, such as nuclease protection or RT-PCR. The variegated RNA classifications are delineated in Table 1.

2.1 Messenger RNA (mRNA)

mRNA molecules are destined to serve as templates for protein synthesis via the action of the translation apparatus in the cell. In eukaryotes, the overwhelming majority of mRNA transcripts are

Tab. 1 RNA types and functions.

RNA type	*Symbol*	*Basic function*	*Prokaryotic*	*Eukaryotic*
Ribosomal RNA	rRNA	Forms back bone of the ribosomal subunits	Yes	Yes
Transfer RNA	tRNA	Transports amino acids to the ribosome to support translation	Yes	Yes
Messenger RNA	mRNA	Template for the synthesis of proteins	Yes	Yes
Heterogeneous nuclear RNA	hnRNA	Large unspliced precursor of mRNA (pre-mRNA)	No	Yes
Small nuclear RNA	snRNA	Facilitates splicing of hnRNA into mature, functional mRNA	No	Yes
Small nucleolar RNA	snoRNA	Processing of immature rRNA transcripts in the nucleolus	No	Yes
Small cytoplasmic RNA	scRNA	Facilitates protein trafficking and secretion	Yes	Yes
Micro RNA	miRNA	Short antisense RNAs that participate in the regulation of gene expression	No	Yes
RNase P RNA	–	Catalytic RNA component of the enzyme/RNA complex that processes tRNA molecules	Yes	No
Telomerase RNA	–	RNA component of the enzyme/RNA complex that repairs chromosome telomeres	No	Yes

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characterized by the presence of a tract of adenosine nucleotides known as the *poly (A) tail*, and all mRNAs so-endowed are known collectively as *poly(A)$^+$ RNA*. As needed, these molecules can be purified from previously isolated cellular RNA, cytoplasmic RNA, or directly from a whole-cell lysate by using affinity chromatography. For this, oligo(dT)$_{12-18}$ linked to one of several popular matrices, including paramagnetic beads, biotin, cellulose beads or microcrystalline cellulose, is used to sequester those transcripts that are polyadenylated. The perceived enrichment is often used to increase the ability to detect very low-abundance transcripts. It is important to note, however, that transcript enrichment performed to increase sensitivity may actually be counterproductive in some cases, because the loss of some mRNA during the enrichment procedure may serve only to further under-represent very low-abundance mRNA. Due in no small measure to the power of the polymerase chain reaction (PCR), and the clever design of the required primers, poly(A)$^+$ selection is viewed by many investigators as unnecessary for most contemporary applications.

Poly(A)$^-$ RNA is that subpopulation of RNA lacking the tract of adenosine residues at the 3′ terminus; it includes a small number of mRNA molecules, a noteworthy example of which are the histone mRNAs. The predominant members of this class, however, include ribosomal RNA (rRNA), transfer RNA (tRNA), and other noncoding transcripts. In instances where poly(A)$^-$ mRNA might not be detected due to exclusion from a sample, either the poly(A)$^-$ fraction or a sample of total RNA from the same biological source will contain these naturally nonadenylated transcripts for assay, assuming their respective genes are transcriptionally active. Moreover, the depletion of poly(A)$^+$ mRNA from a sample renders the resulting poly(A)$^-$ fraction an excellent negative control in the assay of poly(A)$^+$ mRNA species. For all of these reasons, it should be noted that the terms "poly(A)$^+$ mRNA" and "mRNA" are not always synonymous. Finally, mRNAs in eukaryotic cells exhibit an unusual $5' \rightarrow 5'$ linkage between the first two nucleotides, known as the *5′ cap*. This structure not only stabilizes the 5′ end of the transcript but it also efficiently identifies mRNAs as candidates for translation, as these caps are found on mRNAs only, and not on other types of transcripts.

2.2 Transfer RNA (tRNA)

tRNA transcripts are small (74–95 nt) molecules with the responsibility of shuttling amino acids from the cytosol to the aminoacyl site of the ribosome, in order to support the process of *translation*. These tRNAs are not consumed during this process but are simply returned to the cytosol in order to acquire and transport additional amino acid molecules. The cognate amino acid that specific tRNA species will transport is encoded in its anticodon. Although tRNAs are single-stranded molecules, they fold into a characteristic three-dimensional (3-D) clover-leaf shape, and are immediately recognizable.

2.3 Ribosomal RNA (rRNA)

rRNA transcripts form the backbones of the large and small ribosomal subunits. Depending on the organism, as many as 80 or more proteins "decorate" the rRNAs in order to form functional protein-synthesis

Tab. 2 Comparison of the traditional Northern analysis, nuclease protection assay, nuclear runoff assay, and RT-PCR.

Cell type	*Major rRNA species*	*Electrophoretic mobility*	*Subunit sizes*	*Intact ribosome*
Prokaryote	16S, 23S	1.5 kb, 2.9 kb	30S, 50S	70S
Eukaryote	18S, 28S	1.9 kb, 4.7 kb	40S, 60S	80S

factories. In prokaryotes, the small and large ribosomal subunits are known as the *30S* and *50S*, respectively, and their eukaryotic counterparts are known as the *40S* subunits and *60S* subunits, where S represents the "Svedberg unit," which is a sedimentation coefficient.

In the cell, the ribosome subunits are dissociated until just prior to the initiation of translation but, upon the completion of translation the ribosome again separates into its constituent subunits. rRNA is the most abundant type of transcript in the cell, often contributing up to 80% of the total RNA. As such, the major rRNAs species are useful as molecular weight standards for RNA electrophoresis, as indicated in Table 2.

2.4 Nuclear RNA

Nuclear RNA is often studied in conjunction with the independent characterization of cytoplasmic RNA as a means of assessing the level (transcriptional versus post-transcriptional) and the degree of regulation of various genes. It is well documented that a large mass of transcribed RNA is degraded in the nucleus; this precursor RNA never matures into mRNA capable of supporting translation in the eukaryotic cytoplasm. By comparing the nuclear abundance and cytoplasmic abundance of a particular RNA, a cause–effect relationship may be discerned between an experimental manipulation and the regulation of gene expression in that system with respect to RNA biogenesis, because heterogeneous nuclear RNA (hnRNA), produced by the action of the enzyme RNA polymerase II, matures into mRNA. The analysis of nuclear RNA may also be performed in order to determine the *rate* at which genes are transcribed (e.g., in the nuclear runoff assay; see below), as opposed to the assay of steady-state RNA levels; these data can then be used to assess the level of regulation of gene expression.

Small nuclear RNAs (snRNAs) represent another class of nuclear RNA. These molecules typically exist as the RNA–protein complexes, known as U1, U2, U4, U5, and U6, and are confined to the nucleus where they are generically referred to as small nuclear ribonucleoproteins (snRNPs, or "snurps"). snRNPs are now known to form enormous complexes referred to as *spliceosomes*; these have responsibility for the removal of noncoding intron sequences found in hnRNA and concomitant exon ligation during mRNA biogenesis. Yet another class of small nucleolar RNAs (snoRNAs) is associated with rRNA biogenesis in the nucleolar region, where transcription of the rRNA genes occurs.

2.5
Organellar RNA

Both mitochondria and chloroplasts have their own circular chromosomes (mitochondrial DNA, mtDNA and chloroplast DNA, ctDNA, respectively) which are inherited independently of nuclear chromatin, and in a non-Mendelian manner. These unique genomes encode proteins that remain in the organelle, although mitochondria and chloroplasts each import proteins encoded by nuclear genes to support normal organellar function. In contrast to cytoplasmic mRNAs, neither mitochondrial nor chloroplast mRNAs exhibit a 5′ cap structure. Most mitochondrial transcripts exhibit a 3′ relatively short poly(A) tail, while most chloroplast mRNAs are not polyadenylated. Mitochondrial mRNAs often possess unusual AUA and AUU translation start codons, rather than AUG. These start codons are usually observed very close to the 5′ terminus, although there is considerable variation from one cell type to the next.

2.6
Noncoding RNA

Noncoding RNA refers to a population of small transcripts that do not encode proteins but, interestingly, are often intimately involved in the regulation of protein synthesis. This RNA category includes an abundant group of small cytoplasmic RNAs (scRNAs) found in the eukaryotic cytoplasm and, technically, also the well-known rRNA and tRNA species described above. The small cytoplasmic transcripts are known to exist as RNA–protein complexes (scRNP, or "scyrps"), and to have a role in regulating the synthesis, sorting, and secretion of proteins, as well as possible mRNA degradation. Of greatest contemporary interest to the molecular biologist are the microRNAs (miRNAs), which function as noncoding antisense regulators of protein synthesis. The formation of double-stranded RNA (dsRNA) structures via miRNA : mRNA base-pairing (either perfectly or with a mismatch) most commonly occurs near the 3′ end of the cognate transcript, and is able transiently to block the translation of that mRNA, or to direct its destruction altogether.

3
Goals in the Purification of RNA

Concise and thoughtful planning prior to beginning laboratory investigations is an absolute requirement for the recovery of high-quality RNA that is capable of supporting biochemical analyses. During the preliminary stages, an experimental design for the purification of nucleic acids must in general address five specific goals (adapted, in part, from Ref. [1]), the successful achievement of which will have a profound influence on the yield, quality, and utility of the sample.

3.1
Goal 1: Select an Appropriate Method for Membrane Solubilization

The first decision to be factored into an RNA isolation strategy is based on *which* population of RNA or subcellular compartment is to be studied. For example, the aim might be to determine whether an observed modulation of gene expression in a model system is regulated transcriptionally, or by certain post-transcriptional event(s). In such an instance, the methods selected for cellular disruption and

subsequent RNA isolation must permit the analysis of salient nuclear transcripts independently of those localized in the cytoplasm.

The method of cell lysis will determine the extent of subcellular disruption in a sample, and is a direct function of the lysis buffer. For example, a lysis buffer that is used successfully with tissue culture cells may be entirely inappropriate for whole-tissue samples due to the presence of a cell wall (in the case of plants and yeast) or tenacious proteins found in the extracellular matrix (in animal tissues). The method by which membrane solubilization is accomplished will also dictate which additional steps will be required to remove DNA and protein from the RNA preparation, and whether compartmentalized nuclear RNA and cytoplasmic RNA species can be purified independently of one another. While DNA can be purged from an RNA preparation with minimal fanfare, it is not possible to determine the relative contribution of transcripts from the nucleus and from the cytoplasm, once the RNAs from these two subcellular compartments have mingled and copurified. A particular lysis procedure must likewise demonstrate compatibility with ensuing protocols. The main lesson is always to think two steps ahead: the correct method of solubilization is dependent on the plans for the RNA after purification, and the questions being asked of a particular study.

3.2 Goal 2: Ensure Total Inhibition of Nuclease Activity

The imperative for controlling nuclease activity is non-negotiable. This includes purging RNase from reagents and equipment (extrinsic sources of nuclease activity) and controlling the RNase activity in a cell lysate (intrinsic source of nuclease activity). Whilst harsh lysis buffers inhibit nuclease activity in their own right, gentle lysis buffers often require the addition of nuclease inhibitors to safeguard the RNA during the isolation procedure. Steps for the inhibition or elimination of RNase activity must, first and foremost, demonstrate compatibility with the lysis buffer.

3.3 Goal 3: Remove Contaminating Proteins from the Sample

The complete removal of protein from a cellular lysate is of paramount importance in the isolation of both RNA and DNA. Meticulous attention to this detail is required, both for accurate quantification and precision in hybridization, ligation, or reverse transcription into cDNA. The removal of proteins from nucleic acid samples may be accomplished by:

1. Protein hydrolysis with proteinase K
2. Salting-out of proteins
3. Solubilizing proteins in guanidinium-based buffers
4. Repeated extraction with mixtures of phenol and chloroform
5. Any combination of the above.

RNA molecules are much less fragile than high-molecular-weight DNA, and consequently more aggressive methods can be employed for the removal of proteins, including the use of phenol : chloroform extraction. While deproteinization is in itself a means of controlling RNase activity, purified RNA samples will be once again susceptible to nuclease degradation following removal of the protein denaturant, especially as a consequence of latent RNase contamination.

3.4 Goal 4: Concentrate the Sample

This is the final step in nearly all RNA purification schemes. The most versatile method for concentrating nucleic acids is precipitation, using various combinations of salt and alcohol (the most common method is to add sodium acetate and ethanol). Nucleic acids and the salt that drives their precipitation form complexes which have a greatly reduced solubility in high concentrations of alcohol. Unlike the precipitation of genomic DNA, that of RNA typically requires longer incubation periods, often at −20 °C. In addition, when centrifuging samples a greater *g*-force must be applied in order to completely recover an RNA precipitate for subsequent analysis. Other concentration procedures include the use of commercially available concentrating devices, dialysis, centrifugation under vacuum, and binding to silica column matrices in high-salt. Today, silica-based purification formats are widely used and have all but replaced the salt and alcohol precipitation method. In the column format, the purified RNA can be eluted in as small a volume as a few microliters, thereby ensuring a favorably high concentration of nucleic acid that can be used directly. Care must be taken, however, when handling the RNA at this stage of purification, as it will once again be susceptible to nuclease attack when the residual, strongly denaturing lysis buffer components and deproteination reagents have been removed.

3.5 Goal 5: Select the Correct Storage Conditions for the Purified RNA

Because of the naturally labile character of RNA, the incorrect storage of excellent RNA samples will often result in degradation within a relatively short time. Many proposals have been made as to the correct temperature, buffer, and storage form for RNA but, as a general rule, RNA is most stable as an ethanol precipitate at −80 °C. Large samples or RNA stocks should be stored in convenient aliquots in sterile Tris-EDTA buffer (10 mM Tris, pH 7.4; 0.1 mM EDTA) in order to avoid repeated freeze–thaw cycles. Long-term storage in water is not recommended because, over time, the slightly acidic pH environment will favor RNA degradation by acid depurination. Moreover, it is incumbent upon the investigator to ensure that added RNase inhibitors for either long-term or short-term storage will not interfere with any subsequent manipulations and/or reactions involving the RNA.

4 Methods of Cellular Disruption and RNA Recovery

As suggested above, in order to select a suitable method for cellular disruption or "solubilization," consideration must be given as to which subpopulation of RNA is desired for study, as well as the nature of the biological material to be used (cells grown in tissue culture versus whole tissues). Beyond cell and tissue disruption, the absolute necessity for the highest purity, and highest quality, RNA cannot be understated. RNA molecules bind a variety of cytoplasmic and nuclear proteins, any one of which is capable of interfering with most downstream applications, including PCR. Consequently, lysis buffers that effectively strip away RNA-binding proteins are strongly preferred.

The removal of protein during RNA recovery from its biological source often

begins with an application of the lysis buffer. In other cases, the addition of protein denaturants post-lysis is performed, particularly when organellar integrity must be maintained. In either case, thorough attention to this facet of nucleic acids isolation will minimize any subsequent purity-associated problems. While the details of many lysis buffer formulations have been reported, they may all be classified as being either "gentle" or "harsh."

4.1 Gentle Lysis Buffers

Gentle lysis buffers are used when a specific subpopulation of RNA is desired (e.g., cytoplasmic RNA alone) and nuclear integrity must be maintained, as with the isolation of cytoplasmic RNA. Gentle lysis buffers, which often are slightly hypotonic, frequently contain the nonionic detergent NP-40 (Nonidet P-40; today known as *Igepal CA-630*). Because osmotic lysis is the least aggressive method of cellular disruption, NP-40 lysis buffers are ideal for solubilization of the plasma membrane alone, while the inclusion of low concentrations of magnesium helps to maintain nuclear integrity [2]. Thus, the nucleus and its contents (DNA and nuclear RNA) can be separated from the cytosol by using differential centrifugation. The resultant supernatant will be rich in cytoplasmic RNA and proteins, with the latter being easily removed by repeated extraction with phenol : chloroform, or using one of the above-described alternatives. If desired, the nuclear pellet may be processed separately for the recovery of nuclear transcripts. This method of cellular disruption is ideally suited to cells harvested from tissue culture; unfortunately, owing to the complex geometry and formidable nature of whole-tissue samples, nonionic lysis buffers are not effective with tissue samples unless they are coupled with limited, nonshearing homogenization (e.g., using a Dounce homogenizer).

The clear advantage of this isolation strategy is that, ultimately, the material recovered is cytoplasmic RNA alone (mRNA, tRNA, and rRNA). A disadvantage, however, is that the lysis buffer is not sufficiently inhibitory toward RNase. When cell lysis occurs, those RNases which normally are sequestered will be liberated, and their activity will greatly compromise the integrity of the RNA, despite the investigator seeking diligently to maintain its purity. At this point it may be helpful to keep the samples on ice at all times (unless the protocol specifically dictates otherwise); it might also help to use reagents and tubes that have been pre-chilled on ice before use. If desired, an exogenous RNase inhibitor such as RNasin® (Promega) can be added to the lysis buffer. Alternatively, hnRNA (nuclear RNA) alone can be isolated using this same gentle lysis buffer which, when used correctly, does not cause nuclear breakage. This facilitates the recovery of intact nuclei that can be washed free from any residual cytoplasmic transcripts.

4.2 Harsh Lysis Buffers

There is probably no better way to deal with seemingly recalcitrant RNases than to disrupt cells in a guanidinium lysis buffer [3]. On contact, guanidinium-containing buffers distort the tertiary folding of RNases, which results in their inactivation. Other chaotropic lysis buffers which contain high concentrations of ionic detergents, such as sodium dodecylsulfate (SDS), have also been described. The inclusion of additional RNase inhibitors to

these lysis buffers is not necessary, and such procedures for RNA isolation are usually carried out at room temperature.

In the presence of chaotropic agents, organelle lysis accompanies disruption of the plasma membrane. Thus, nuclear RNA, genomic DNA and mitochondrial DNA will all be copurified with cytoplasmic RNA, such that additional steps will be required to remove the DNA from the sample. In the past, the most prevalent of these methods was isopycnic centrifugation [4], which involved gradient centrifugation using either cesium chloride (CsCl) [5] or cesium trifluoroacetate (CsTFA) [6]. Isopycnic separation of the biomolecules is possible because of their differing buoyant densities (DNA, 1.5–1.7 g ml^{-1}; RNA, 1.7–2.0 g ml^{-1}; protein, 1.1–1.2 g ml^{-1}).

The differential partitioning of DNA, RNA and protein by acid–phenol extraction, which was first described by Chomczynski and Sacchi [7], led to a dramatic change in the way that RNA (in particular) could be purified from cells and tissues. Succinctly, the organic extraction of nucleic acids at acidic pH causes DNA to partition to the interphase and organic phase, while RNA remains in the aqueous phase. This approach precludes the requirement for ultracentrifugation, and thus greatly reduces the required amount of hands-on time, to the obvious benefit of the investigator. The popularity of ''acid–phenol extraction'' has resulted in the development of a number of nucleic acid isolation reagents that support the unceremonious purification of RNA from both tissues and tissue cultured cells alike.

In order to take full advantage of the disruptive nature of the guanidinium isolation procedures, whilst maintaining the subcellular compartmentalization of RNA, one worthwhile strategy is to start the isolation procedure with gentle nonionic lysis, followed by the recovery of intact nuclei, which are then lysed with guanidinium buffer. The purification of nuclear (or cytoplasmic) RNA then proceeds as if working with intact cells. This approach is particularly suited to the isolation of nuclear RNA for Northern analysis.

The principal drawback when applying these chaotropic methods to intact cells is the loss of any ability to discriminate between cytoplasmic and nuclear RNA. There is no method by which nuclear RNA can be separated from mRNA once mixing has occurred, although size fractionation may result in a partial separation. Moreover, it is unfortunate that many seasoned investigators begin to show signs of sloppiness with respect to the control of RNase activity when working routinely with guanidinium buffers. Whilst it is true that RNA is safe from nuclease degradation in the presence of these agents, the purified RNA is once again susceptible to nuclease degradation.

4.3
Silica Separation Technology

One of the more important improvements in the area of nucleic acid isolation has been the development of silica filters that are small enough to be used with a standard microcentrifuge. The filters consist of glass microfibers positioned in the bottom of small plastic insert that fits inside a standard 1.5 ml microfuge tube. The filters are widely available, and may be used for the efficient purification of RNA directly from biological sources. They can also be used to clean up nucleic acids after restriction enzyme digestion, ligation reactions, cDNA synthesis, and PCR amplifications. In general, the RNA (or DNA) is bound to silica in a high-salt, chaotropic environment that is produced by diluting a nucleic

acid sample in guanidinium thiocyanate. Following a series of washes, the purified material is eluted from the matrix under very low-salt conditions. The main benefit of this procedure is that the nucleic acid purification and clean-up can be performed within a remarkably short time, and using small volumes.

4.4 Affinity Matrices

In addition to the methods described above for the isolation of total cellular RNA or total cytoplasmic RNA, certain products are available which capture polyadenylated transcripts directly. For example, many mRNA isolation kits feature tracts of oligo(dT) that have been linked covalently to a solid support such as cellulose, polystyrene, latex, or paramagnetic beads. The polyadenylated transcripts are then captured through canonical base-pairing between the poly(A) tail and the oligo(dT) tract in a high-salt environment. The main benefit associated with affinity selection is an enrichment of a nucleic acid sample in favor of mRNA by minimizing the carry-over of rRNA and tRNA; enrichment in this manner may also increase the sensitivity of an assay. An older variant of affinity selection involved poly(A)$^{+}$ mRNA being affinity-captured by using a column packed with poly(U) linked to Sepharose beads [8]. Although still available, this process is no longer generally used because of a perception that is a less-efficient matrix, and that the quantitative recovery of RNA from a poly(U) matrix normally requires the use of formamide-based elution buffers.

Yet another variant of the affinity matrix approach is designed to study nucleic acid–protein interactions by passing a heterogeneous protein mixture over a column packed with either RNA or DNA oligonucleotides, in order to capture proteins with some level of binding affinity to the sequences on the column. The nucleic acid is often referred to as the *"bait"*, while the proteins that can bind to it are known as the *"prey"*. The procedure, which may be referred to as a *"pull-down" method*, is still popular for the characterization of RNA- or DNA-binding proteins, despite the advent of glass or plastic arrays (also known as "chips") that can be used for proteome profiling.

5 Inhibition of Ribonuclease Activity

The difficulties associated with the isolation of full-length, intrinsically labile RNA are further compounded by ubiquitous RNase activity. The RNases are a family of enzymes which degrade RNA molecules through both endonucleolytic and exonucleolytic activity cleavage. These small, remarkably stable enzymes resist denaturation under harsh conditions such as extremes of pH and autoclaving that would easily destroy the activity of many other enzymes [9]. It is incumbent upon the investigator to ensure that both the equipment and the reagents to be used are purged of nucleases from the onset of an experiment. For most RNA-minded molecular biologists, to say that a reagent or apparatus is sterile is more than likely a statement that it is RNase-free.

The method selected for controlling the RNase activity must, first and foremost, demonstrate compatibility with the cell lysis procedure. Occasionally, nuclease inhibitors are added to gentle lysis buffers when subcellular organelles (nuclei especially) are to be purified intact, as in the partitioning of nuclear RNA

from cytoplasmic RNA. However, keeping the reagents and microfuge tubes ice-cold throughout the procedure is also an effective means of controlling nuclease activity. Second, the method of nuclease inhibition must support the integrity of the RNA throughout the subsequent fractionation or purification steps. Third, the reagents used to inhibit the RNase activity must be easily removed from the purified RNA, so as not to interfere with any subsequent manipulations. In all cases – and especially when characterizing a system for the first time – the control of nuclease activity should be aggressive. Failure to do so is likely to yield a useless sample of degraded RNA.

5.1 Preparation of Equipment and Reagents

Rule number one when working with RNA is to wear gloves during the preparation of reagents and equipment, and especially during the actual RNA extraction procedure. Finger greases are notoriously rich in RNase, and are generally accepted as the single greatest source of RNase contamination. There should be no hesitation in changing gloves several times during the course of an RNA-related experiment. Door knobs, micropipettors, computer keyboards, iPods, refrigerator door handles, containers in which chemicals are packaged, and other unassuming surfaces are all potential sources of nuclease contamination.

With respect to laboratory consumables, any plasticware that is certified as being tissue culture-sterile is always preferred when working with RNA. This includes individually wrapped serological pipettes and conical 15 and 50 ml tubes. In any event, these items should be handled only when wearing gloves. Bulk-packed polypropylene products (e.g., microfuge tubes and micropipette tips) are potential sources of nuclease contamination, due mainly to their being handled and distributed with ungloved hands from a single bag. These consumables are best purchased as being certified both DNase- and RNase-free. Any plastic product or other implement that will come into contact with an RNA sample at any time, either directly or indirectly, and which can withstand autoclaving, should be so treated and set aside exclusively for RNA studies.

When the use of glassware is unavoidable (as when using organic reagents such as phenol and chloroform), the use of individually wrapped borosilicate glass pipettes is strongly preferred. Any glassware that must be re-used should be set aside for RNA work, and not allowed to enter general circulation in the laboratory. Contrary to popular belief, the temperature and pressure generated during the autoclaving cycle are usually insufficient to eliminate all RNase activity. Fortunately, however, RNases can be destroyed quite effectively by baking in a dry heat oven; glassware to be used should be rinsed with RNase-free water and then baked for 3–4 h at 200 °C. Baking pertains to glassware alone; any problems regarding the heating of plastics or other materials can usually be resolved by the manufacturers' technical department. Finally, it is vital to pay attention to the expiry dates of all compounds and solutions in the laboratory. Older bottles of stock solutions in particular serve as excellent breeding grounds for microorganisms, which shed their RNase into the solution. The use of such a contaminated stock solution could lead to the obliteration of an entire RNA sample.

5.2 Inhibitors of RNase

Endogenous RNase activity varies tremendously from one biological source to the next, and the degree to which action must be taken to inhibit nuclease activity is a direct function of the cell type. Knowledge of the extent of intrinsic nuclease activity is derived from two principal sources: the salient literature, and personal experience. The method of RNase inhibition is to a great extent a function of the type of lysis buffer. Whereas, nondenaturing, osmotic lysis buffers often include a nuclease inhibitor, strongly denaturing (chaotropic) lysis buffers generally do not. Such chaotropic compounds include guanidinium thiocyanate, guanidinium HCl, sarcosyl, SDS, 8-hydroxyquinoline, CsCl, CsTFA, and/or various formulations of organic solvents.

RNasin® may be used to inhibit nuclease activity and circumvent some of the problems commonly associated with the use of a vanadyl ribonucleoside (VDR) complex, and is compatible with a variety of *in vitro* reactions. RNasin® inactivates RNase A, RNase B, and RNase C, but not RNase T1, S1 nuclease, nor RNase from *Aspergillus*. Care must be taken to avoid any strongly denaturing conditions that will cause the uncoupling of RNase – RNasin® complexes and the reactivation of RNase activity. RNasin® is widely used in reverse transcription reactions in order to protect the integrity of the template RNA prior to the synthesis of first-strand cDNA.

At one time, a VDR was a popular addition to nonionic lysis buffers which alone are ineffective for the control of RNase. In the absence of a VDR, the RNase-mediated cleavage of the phosphodiester backbone of RNA results in the transient formation of a dicyclic transition state intermediate that is subsequently opened by reaction with a water molecule. In its capacity as an RNA transition state analog, the VDR complex forms a highly stable dicyclic species to which the enzyme remains irreversibly bound. Thus, nuclease activity is eliminated by locking RNase and ‘‘pseudo-substrate’’ in the transition state. The VDR binds tightly to a broad spectrum of cellular RNases, including RNase A and RNase T1, but not to RNase H, and is compatible with a variety of cell fractionation methods. It is important that a VDR is used selectively, however, as even trace carry-over quantities are sufficient to inhibit the *in vitro* translation of purified mRNA. It can also interfere with reverse transcriptase activity, thereby excluding its use with any RT-PCR applications. For this reason, the VDR is no longer used by most molecular biologists as an RNase inhibitor.

Diethyl pyrocarbonate (DEPC), which at one time was used widely to purge RNase from solutions prepared in-house, has also fallen out of favor with molecular biologists. This is due to the widespread availability of certified nuclease-free reagents, including sterile H_2O, from virtually all biotech vendors. DEPC is a well-known nonspecific inhibitor of RNase that is used to purge reagents of nuclease activity, due to the unreliability of autoclaving alone. Strict precautions (as indicated by the manufacturer) must be taken when using DEPC, however, as it is carcinogenic and potentially explosive. Clearly, it should be avoided unless there is an absolutely compelling reason for its use.

Hydrogen peroxide (H_2O_2) is a powerful oxidizing agent that can render common laboratory surfaces nuclease-free by soaking for 20–30 min, followed by rinsing with copious amounts of water that, at

the very least, has been autoclaved. The soaking of glass pipettes, gel box casting trays, electrophoresis combs, graduated cylinders, and similar implements in a 3% H_2O_2 solution is a very effective and inexpensive measure. H_2O_2 is readily available in pharmacies and similar stores. It is important NOT to use the more concentrated forms of H_2O_2 (e.g., 30% H_2O_2) that are commonly available from chemical supply companies since, at this higher concentration H_2O_2 is extremely dangerous, perhaps causing irreparable damage to acrylic gel box components and other equipment, as well as tissue damage to the investigator. Old solutions of H_2O_2 must also be avoided, as they may no longer be solutions of H_2O_2!

Since many RNases manage to renature following removal of the denaturing reagent(s), it is prudent to maintain separate containers of chemicals and stock solutions for exclusive use as RNA reagents. Chemical solids should be weighed out with an RNase-free spatula, while stock solutions should be aliquoted into suitable volumes; any aliquots that have been used must be discarded. While, initially, such actions may seem excessive, they may well preclude the accidental introduction of RNase and facilitate an expedient recovery of high-quality RNA. All laboratories should have established standard operating procedures (SOPs) in place regarding RNA-related studies, and these protocols should be followed meticulously.

6 Methods for the Analysis of RNA

The evaluation of gene expression by the hybridization of RNA is possible in a variety of formats, as is the analysis of DNA. Methods range from the traditional to the contemporary, with each procedure having an applicability under a defined set of experimental conditions, as well as a characteristic level of sensitivity. The relative merits of four such standard methods are listed in Table 3.

6.1 RT-PCR

The PCR is a primer-mediated, enzymatic method for the quasi-exponential amplification of nucleic acid sequences. This method requires any one of several thermostable DNA polymerases, two short oligonucleotides acting as nucleic primer sequences, a dNTP cocktail, and the appropriate chemistry to support the activity of the enzyme. The primers are designed to base-pair to opposite strands of the DNA template with their respective 3′-OH ends facing each other. This leads to the amplification of that sequence which is framed by the 5′ ends of the respective primers through a series of heating, cooling, and primer extension stages, the mechanics of which are discussed in great detail elsewhere in the Encyclopedia of Molecular Cell Biology and Molecular Medicine (EMCBMM).

RT-PCR is a two-step process. First, high-quality RNA acts as the template for the synthesis of first-strand cDNA with the enzyme reverse transcriptase. The components and mechanics of this reaction are almost identical to any other first-strand cDNA synthesis reaction, an example being the construction of a traditional cDNA library. Second, the products of the first-strand synthesis reaction are then amplified using the PCR. Traditionally, the first-strand synthesis products are added to a second tube which provides all of the cofactors necessary to support

Tab. 3 Comparison of the traditional northern analysis, nuclease protection assay, nuclear runoff assay, and RT-PCR.

	Northern analysis	***Nuclease protection assay***	***Nuclear runoff assay***	***RT-PCR***
Advantages	Provides a qualitative component to RNA analysis. Nylon filters support several rounds of hybridization with different probes. Is compatible with total, cytoplasmic, or poly(A)$^+$ RNA. RNA is relatively stable on filter. Is able to assess integrity of the sample.	Higher sensitivity than Northern analysis. Requires less handling of RNA than other types of analysis. Is tolerant of partially degraded RNA. Solution hybridization is more quantitative than filter hybridization. Can be used for steady-state or transcription rate assays.	Characterizes relative rate of transcription. Natural geometry of the chromatin is maintained. Permits simultaneous study of several genes. Can be used to discern transcriptional versus post-transcriptional gene regulation when used in conjunction with data from Northern analysis.	Provides unparalleled sensitivity when properly designed. Provides unparalleled resolution. Supersedes many of the classical techniques. Minimizes the amount of handling of the RNA. Very rapid technique. Favors research productivity
Disadvantages	Is the least sensitive assay. Denaturants can be toxic. Requires extensive handling of RNA. Is a time-consuming process. Provides ample opportunity for RNase degradation. Characterizes only steady-state RNA.	Protected fragment is smaller than native RNA. Nucleases, especially S1, can be difficult to control. Assay is more sensitive to exact hybridization parameters than other assays. Double-stranded probes can compromise quantitativeness of the assay if reannealing occurs.	Nuclear isolation requires a fair amount of skill. Probe complexity is very large. Unlabeled endogenous RNA can compete with labeled RNA during hybridization. Mechanics of the assay support transcript elongation, and not initiation, during labeling.	Much more sensitive to the precise reaction components and conditions than the other assays. Exquisitely sensitive to contaminants, especially genomic DNA. Carry-over contamination must be addressed. Optimization can be time-consuming and costly.

Reproduced with permission from Farrell, Jr, R.E. (2010) *RNA Methodologies: A Laboratory Guide for Isolation and Characterization*, 4th edn, Elsevier, p. 344 [1].

the amplification of these products by PCR. A more recently developed method for performing RT-PCR, which is now widely used in clinical and diagnostic laboratories, requires only one enzyme in a single reaction tube format (one-tube RT-PCR). In either case, the newly synthesized cDNA is amplified as would be the DNA from any other source, predicated upon the availability of a set of gene-specific primers. The PCR-amplified cDNA products can then be quantified, or in some other way analyzed, in order to more fully understand some aspect of normal, or abnormal, cell function. RT-PCR is advantageous because the very labile character of RNA does not favor its long-term storage. The synthesis of cDNA provides a template for a DNA polymerase-mediated amplification on an immense scale; only those transcribed RNAs which are purified from the cell can be converted into cDNA. Different tissues – even from the same biological source – will yield different cDNA products, such that cDNA may be best thought of as a permanent biochemical record of the cell. cDNA represents a means by which the molecular physiology of the cell can be studied in great detail over a period of months or years – much longer, and with much greater sensitivity, than the assay of purified RNA directly.

In addition to its obvious utility for the quantification of gene expression, the judicious design of primers permits RT-PCR to be used to map the 5′ and 3′ ends of transcripts – a method known as the rapid amplification of 5′ complementary DNA ends (5′ RACE) [10] and the rapid amplification of 3′ complementary DNA ends (3′ RACE) [11], respectively. RACE is used to detect alternative transcript initiation, splicing, and poly(A)$^+$ polymerization sites, and to identify induced and repressed genes under a defined set of environmental conditions.

Finally, RT-PCR can be performed using two different platforms, namely end-point PCR and real-time PCR; the latter method may also be referred to as the quantitative polymerase chain reaction (qPCR). End-point PCR involves amplifying the template over 25–30 cycles, with a theoretical amplification of 2^n-fold, where n is the number of cycles. When all of the cycles have been completed, the reaction tube is opened and the resulting products are analyzed using agarose gel electrophoresis. In this case, the band intensity is associated with product abundance, which in turn mirrors the abundance of the starting material. Both, the mechanics of end-point PCR and the method of detection can limit the sensitivity of end-point PCR. For example, the intensity of two bands representing two vastly different samples may appear identical on electrophoresis when one reaction depletes the primers (the so-called ''plateau effect'') after 15 cycles, and the other reaction depletes the primers after 30 cycles.

Real-time PCR is widely regarded as the ''gold standard'' with respect to nucleic acid detection sensitivity. In the real-time format, the accumulation of product in the reaction vessel is measured at the end of every cycle – that is, in real-time. As the PCR product accumulates, however, there will be a directly proportional increase in fluorescence, due to the inclusion of fluorescent precursors in the reaction chemistry. The fluorescence detection system permits an extremely early detection in the amplification process, while the accumulation of product is reliably exponential. With each passing cycle, however, inefficiencies in the reaction itself compromise the amplification efficiency of subsequent cycles. As a consequence, the true abundance

relationships among genes and among samples may be distorted, or even lost altogether, by waiting until the end of all cycles before the products are analyzed. Moreover, the fact that real-time quantification occurs in a sealed tube that is not opened at all greatly minimizes the risk of carry-over contamination – an unfortunate occurrence where the product from one PCR experiment inadvertently becomes the template in a subsequent experiment.

It is also important to note that, following recovery from the cell, intramolecular base-pairing that results in secondary and tertiary RNA structures is problematic. Molecules in which higher-level structures form are often resistant to reverse transcription, which thereby diminishes their ability to be quantified or otherwise assayed. This issue is often addressed by heat denaturation in the presence of one or more compounds that interfere with hydrogen bonding, and is performed prior to reverse transcription. Further, performing the first-strand cDNA synthesis reaction at elevated temperatures also helps to reduce any intramolecular base-pairing; this is possible because of the availability of thermostable reverse transcriptases.

6.2 Northern Analysis

The quintessential method for the assay of gene expression is a method referred to as *Northern analysis* [12] (it is also known colloquially as Northern blotting, the Northern blot analysis, and/or RNA blot analysis. Northern analysis involves the electrophoretic separation of RNA molecules under denaturing conditions, with subsequent transfer or ‘‘blotting’’ of the sample onto a solid filter support (the so-called ‘‘filter membrane’’). The RNA on the blot is then hybridized to an appropriately labeled nucleic acid probe which will support subsequent detection by autoradiography, or by chemiluminescence. Because the samples of RNA undergo electrophoresis prior to their hybridization, the Northern analysis provides both quantitative and qualitative biochemical profiles of the sample. Denaturation of the RNA prior to electrophoresis is necessary to ensure that the migration of the sample through the gel occurs only with respect to molecular weight, and is not distorted by the formation of any secondary structure that is commonly associated with single-stranded molecules. Thus, the length of the transcript(s) can be determined – a datum that cannot be discerned using other methods.

The objective of the Northern analysis is to quantify gene expression by detecting the relative abundance of those mRNAs in the sample which are of immediate interest to the investigator. Whereas, in the Southern analysis [13] the resulting data pertains to the structure and organization of genes, data derived by Northern analysis reflects the transcriptional activity of genes.

The principal shortcoming associated with Northern blot data is the limited sensitivity of the assay. The physical application and immobilization of an RNA sample onto a filter membrane renders some of those molecules incapable of base-pairing to a complementary nucleic acid probe. Neither is the Northern analysis intended to discern the absolute mass of RNA in the cell. Rather, such data may be measured far more accurately by using solution hybridization-based methods, especially real-time PCR. Hence, data derived from the Northern analysis must be interpreted in the context of the relative abundance of a particular RNA among all

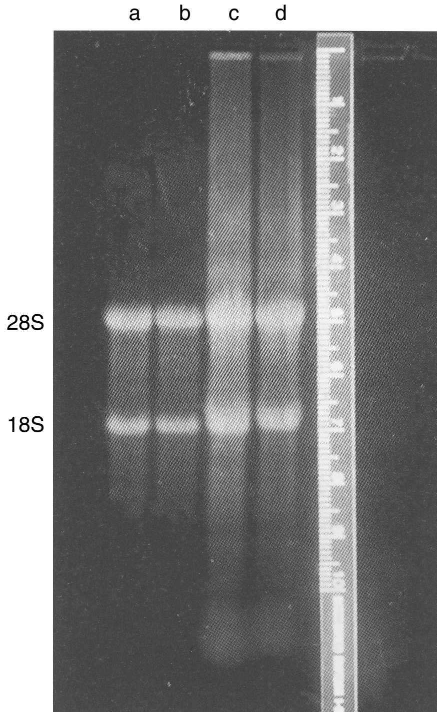

Fig. 1 Assessment of RNA quality. The sharp definition of the 28S and 18S rRNA species in lanes a and b demonstrates the integrity of the sample. RNA in lanes c and d is also high quality, although an excessive amount of RNA was applied to these lanes. Lanes a and b: 20 µg of total cytoplasmic RNA prepared by NP-40 lysis. Lanes c and d: 25 µg of total cellular RNA (nuclear and cytoplasmic), prepared by guanidinium–acid–phenol extraction. Note the higher molecular weight nuclear RNA species in the sample. Reproduced with permission from Farrell Jr, R.E. (1993) *RNA Methodologies: A Laboratory Guide for Isolation and Characterization*, Academic Press, San Diego, CA, p. 60).

of the samples involved; hence, the method is semi-quantitative at best.

The electrophoresis of RNA is itself an important diagnostic tool, with a host of information being made available regarding the integrity and probable utility of an RNA sample by examining a representative aliquot. RNA has a highly characteristic profile on a denaturing gel (Fig. 1), whereby the appearance of the predominant species – the 28S and 18S rRNAs – being an indicator of the integrity of the sample. Ideally, a very light smearing above, between, and just barely below the rRNAs indicates that sample is intact and is probably capable of supporting nucleic acid hybridization. Heavier smearing, especially below the level of the 18S rRNA is quite ominous, being indicative of partially or fully degraded RNA (Fig. 2). The complete absence of the rRNAs indicates a completely degraded sample. As it is clearly desirable to ascertain the integrity of a sample before moving on to sophisticated and often time-consuming techniques, a brief period of electrophoresis to assess the quality of the sample should become

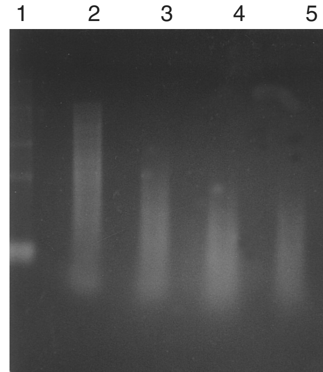

Fig. 2 Going, going, gone ... degraded RNA. A representative aliquot from four different samples of human fibroblast RNA was electrophoresed in a 1.2% agarose-formaldehyde gel and then stained with ethidium bromide. The RNA molecular weight standard is visible in lane 1. RNA in lanes 2–5 shows increasing degrees of degradation, most likely due to RNase contamination during the isolation procedure. Especially noteworthy is the complete absence of the 28S and 18S rRNA species expected in high-quality RNA. This is an excellent example of what not to do. Reproduced from with permission from Farrell Jr, R.E. (2010) *RNA Methodologies: A Laboratory Guide for Isolation and Characterization*, 4th edn, Elsevier, Academic Press, San Diego, CA, p. 149) [1].

a standard procedure in any molecular biology setting.

6.3 Nuclease Protection Assay

The intrinsic shortcomings of the Northern analysis mandate a different format for the assay of gene expression when very exacting quantitative data are required. In contrast to the assay format of the Northern analysis, at the heart of an assay by nuclease protection is a high stringency hybridization between the target and probe molecules, both of which are free-floating in solution (solution hybridization) as opposed to having the target mRNA fixed on the filter paper (mixed-phase hybridization). The driving forces behind solution hybridization are the random molecular collisions, the kinetics of which are related directly to the total mass of nucleic acid in the reaction tube (probe + target + carrier = total mass). Because of the solution hybridization format, all complementary nucleic acid molecules are presumed to be capable of hybridization. The S1 nuclease protection assay (Fig. 3) and the RNase protection assay (Fig. 4) are methods of greatly enhanced sensitivity and resolution, and are universally considered to be more quantitative than Northern analysis.

The best nucleic acid probes for these assays are substantially shorter than the target mRNA. Upon molecular hybridization, a short double-stranded region is generated, while the 5′ and 3′ regions of the target molecule flanking the double-stranded area remain single-stranded. The enzyme S1 nuclease, or a combination of RNases, is then used to digest all of the nucleic acid molecules that did not participate in nucleic acid hybridization. Only double-stranded nucleic acid molecules are resistant to

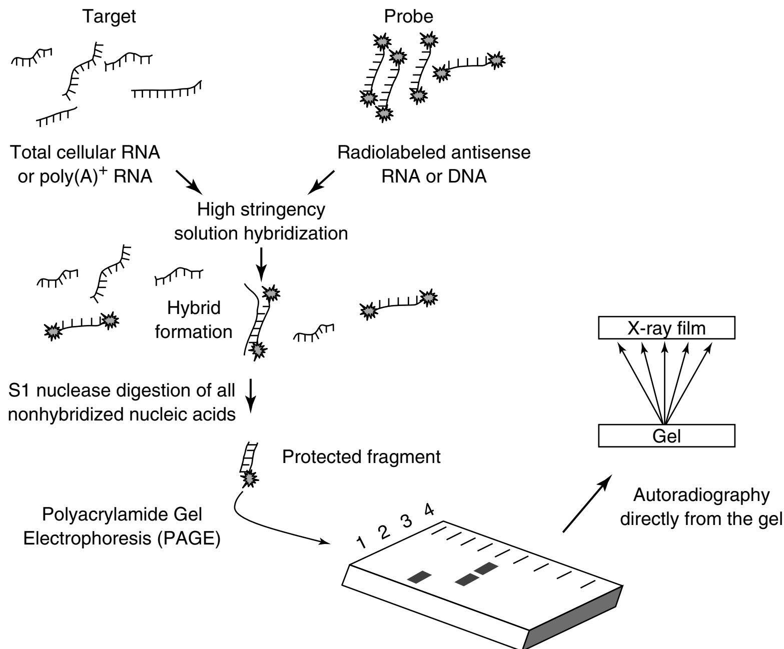

Fig. 3 S1 nuclease assay for the quantification of specific RNA species. Purified RNA is hybridized in solution with a labeled probe sequence to form thermodynamically stable hybrid molecules. Any RNA or probe molecules that do not participate in the formation of hybrid molecules are digested away by the single-strand-specific nuclease S1, followed by electrophoresis of the intact hybrid molecules. The size and abundance of protected RNAs are then deduced by autoradiography, performed directly from the gel. Lane 1: undigested probe; lanes 2 and 3: experimental samples; lane 4: molecular weight standards. Reproduced with permission from Farrell Jr, R.E. (2010) *RNA Methodologies: A Laboratory Guide for Isolation and Characterization*, 4th edn, Elsevier, Academic Press, San Diego, CA, p. 323) [1].

nuclease attack. The resulting product of this assay – the so-called "protected fragment" – is then resolved by electrophoresis. By virtue of the mechanics of this assay, the size of the protected fragment is expected to be similar to the size of the probe sequence itself, which is often substantially shorter than the native RNA target, and can be visualized by using autoradiography. As a direct result of solution hybridization and the digestion of all nonhybridized nucleic acid molecules, the investigator can expect an at least 10-fold enhancement in sensitivity, compared to Northern analysis, particularly when performed using antisense RNA probes.

6.4 Transcription Rate Assays

The modulation of key regulatory molecules is an integral cellular response to both intracellular and extracellular challenge. One fundamental goal in the assessment of any biological model system is an elucidation of the level of gene modulation. While potential levels of regulation are infinite, they are

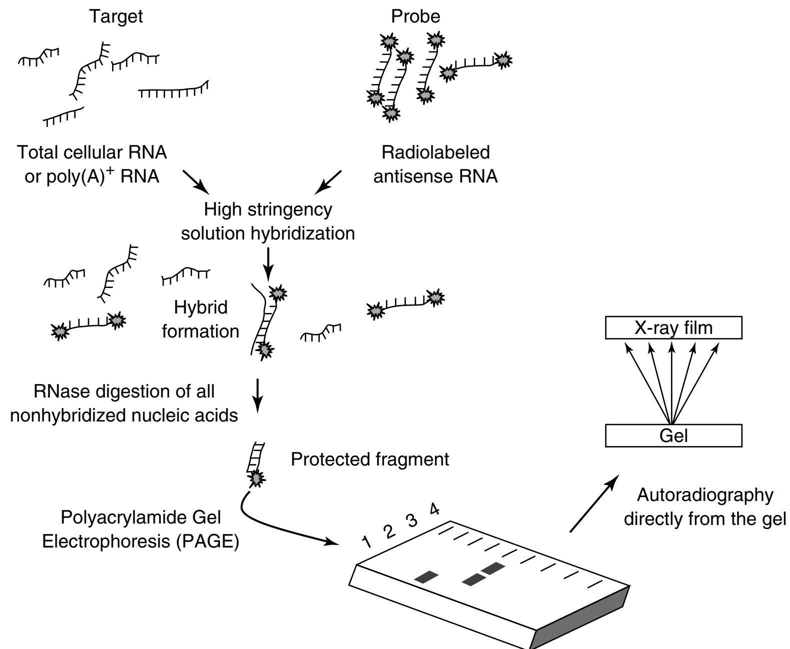

Fig. 4 RNase protection assay for the quantification of specific RNA species. Purified RNA is hybridized in solution with a labeled antisense probe sequence to form thermodynamically stable double-stranded RNA molecules. Any RNA or probe molecules that remain single stranded are digested by an RNase cocktail. Following electrophoresis, the size and abundance of protected RNAs are then deduced by autoradiography, performed directly from the gel. Lane 1: undigested probe; lanes 2 and 3: experimental samples; lane 4: molecular weight standards. The general approach is identical to that for the S1 nuclease assay. Reproduced with permission from Farrell Jr, R.E. (2010) *RNA Methodologies: A Laboratory Guide for Isolation and Characterization*, 4th edn, Elsevier, Academic Press, San Diego, CA, p. 324) [1].

broadly categorized as transcriptional or due to some post-transcriptional event. The initial characterization of these systems commonly involves the isolation, hybridization and subsequent detection of specific RNA species by RT-PCR, nuclease protection analysis, or even Northern analysis. While these approaches may provide reliable qualitative and quantitative data with respect to steady-state levels of message, RNA prepared by total cellular lysis does not provide information about the *rate* of transcription, the subcellular compartmentalization (nuclear or cytoplasmic) of the RNA under investigation, or the translatability of the RNA in the cytoplasm. Knowledge of these aspects of gene expression is necessary to elucidate the level of gene regulation, because the half-lives among RNA species are variable and because the half-life of many mRNA species can be modified in response to a particular xenobiotic regimen or environmental stimulus.

In order to address these questions, two basic approaches have been employed to study the mechanism of transcription and the processing of the resulting transcripts

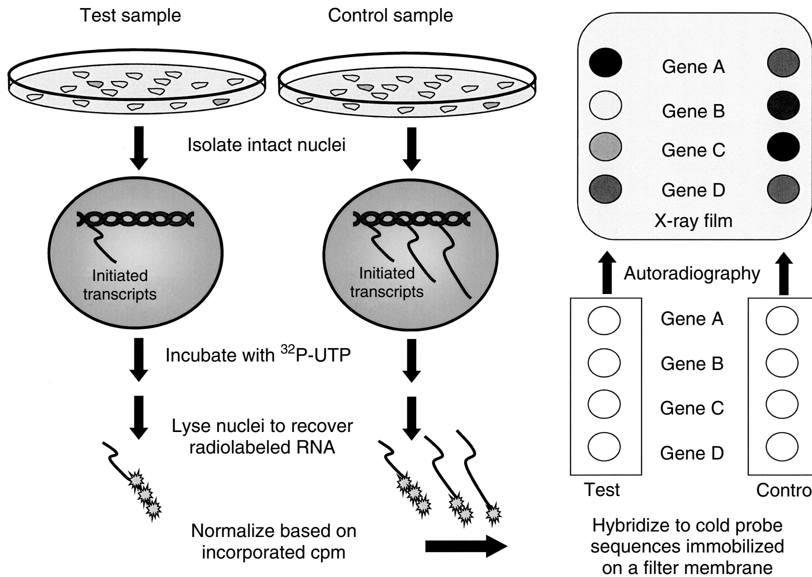

Fig. 5 Nuclear runoff assay. The relative rate of transcription of all genes can be assessed by incubation of intact nuclei with an NTP cocktail containing labeled UTP. Elongated, radiolabeled transcripts are then hybridized to nonradioactive cDNA probes immobilized on a nylon filter. On autoradiography, the intensity of the signal from each dot is indicative of the degree of label incorporation, and thus the relative rate of transcription of specific genes under a define set of experimental conditions. Reproduced with permission from Farrell Jr, R.E. (2010) *RNA Methodologies: A Laboratory Guide for Isolation and Characterization*, 4th edn, Elsevier, Academic Press, San Diego, CA, p. 343) [1].

in eukaryotic cells. In one approach, the rate of transcription is measured in intact nuclei by the incorporation of labeled precursor nucleotides into RNA transcripts initiated on endogenous chromatin at the time of nuclear isolation. Elongated, labeled nuclear RNA is then purified for hybridization to complementary, membrane-bound DNA sequences. This technique, which is known as the *nuclear runoff assay* (Fig. 5), is a superbly sensitive method for measuring transcription rate as a function of cell state [14, 15], and consequently is widely used. Because it is the RNA transcripts, rather than the probes used to quantify their abundance, that are radiolabeled the basic format of this assay can be likened to a "reverse dot-blot," as the probe is membrane-bound and nonradiolabeled.

The principal advantage of the nuclear runoff assay is that labeling occurs whilst maintaining the natural geometry of the transcription apparatus. The mechanics and reaction conditions of the assay promote the elongation of initiated transcripts, but are not believed to support new initiation events. The degree of labeling of any particular RNA species, which is indicative of the relative transcription rate of a specific gene, may then be assessed by liquid scintillation counting (a specific type of radioactive

detection), coupled with autoradiography. These data correlate directly with the number of RNA polymerase molecules engaged in transcribing a specific gene, and indirectly with the transcriptional efficiency of regulatory sequences associated with the gene under a defined set of experimental conditions. When used in conjunction with a steady-state analysis of cytoplasmic RNA species, data from the nuclear runoff assay may be used to assess whether an observed gene modulation is a result of a change in the synthesis (transcriptional control) or a change in the splicing/nucleocytoplasmic transport/ mRNA stability (post-transcriptional control).

The nuclear runoff assay permits the simultaneous analysis of several genes, all of which are presumably transcribed in isolated nuclei at the same relative rates as in intact cells. The most critical parameter by far is the preparation of nuclei prior to labeling. Indeed, the success of the assay is almost entirely dependent on the speed with which nuclei are harvested and radiolabeled with the precursor, uridine triphosphate (UTP). Failure to generate high-specific activity RNA is usually a direct result of inexperienced handling of the nuclei prior to the labeling step. The nuclei are most often isolated by incubating the cells in a nonionic, hypotonic lysis buffer, in isoosmotic sucrose buffer containing Triton X-100, or by using non-aqueous methods [16]. The nuclear purification must also be carried out in such a way as to preserve RNA polymerase activity and nuclear structure during the isolation from cells cultured *in vitro* or, if absolutely necessary, from tissue. The harvested nuclei, if not labeled immediately, may be stored frozen either in liquid nitrogen or at −70 °C in a freezer for several months, without any significant loss of labeling potential.

6.5 Dot-Blot Analysis

The isolation of high-quality RNA from tissue culture cells and whole-cell samples is merely the first (albeit the most critical) step in the evaluation of a model system. Procedures such as Northern analysis, nuclease protection analysis, and conversion into cDNA can be a time-consuming and expensive option, and should not immediately be deemed necessary. When evaluating a model system, cell type, or experimental regimen for the first time, it may be worthwhile quantifying the mRNAs of interest by using a dot-blot analysis. This simple technique allows definitive statements to be made regarding the biochemical composition of a sample, but without investing excessive man-hours and laboratory resources.

In order to dot-blot RNA, denatured samples are applied directly onto a membrane under vacuum, using a multi-well dot-blot filtration manifold (Fig. 6). The samples are immobilized onto the surface of the membrane, followed by nucleic acid hybridization. In this way, dot-blots and a closely related variant known as *slot-blots* permit the rapid detection of the relative amounts of a particular RNA in a sample. Salient information can be obtained from purified RNA samples or whole-cell lysates, without performing electrophoresis or any form of PCR. These methods are reserved for the later, more exacting analysis of a system after the preliminary information has been derived.

Dot-blotting represents an excellent method for the analysis of gene expression

Fig. 6 Minifold I dot-blot apparatus. Sample dilutions are applied under vacuum directly to the surface of the filter membrane resting beneath the face plate. The geometric arrangement of the samples allows easy visual examination of several samples and also facilitates digital image analysis. Photograph courtesy of Schleicher & Schuell, Inc., Keene, NH.

when large numbers of samples are to be evaluated simultaneously, such as an experiment requiring numerous time points. If sample dilutions are desired, they may be arranged either vertically or horizontally, and the degree of hybridization can then be assessed using image analysis software.

The two main drawbacks of the dot-blot analysis, which yields purely quantitative data, are: (1) that it lacks the qualitative component that accompanies electrophoresis; and (2) that the immobilization of the samples on a membrane severely limits the assay's quantitative character. In order to be truly reliable, a dot-blot analysis must include excellent positive and negative controls to demonstrate hybridization specificity, and to gauge any nonspecific binding of the probe to the filter membrane. Moreover, good internal controls are always in order: equally intense signals should be observed from wells into which equal amounts of positive control target have been applied. When attempting this type of blot analysis for the first time, or when using a new system, it is strongly suggested that dilutions of the positive control target material are made in order to determine the linear range of the assay. For example, it would be useless – quantitatively speaking – if the hybridization signals were too intense to be accurately measured on X-ray film, which also has a defined linear range [17].

6.6 High-Throughput Transcription Analysis

The ability to rapidly screen a large number of samples and simultaneously assay the expression of as many genes as possible (global analysis of gene expression) has become a reality with the development of microarrays. The observed pattern of a large number of genes that are modulated under a defined set of conditions, which sometimes is referred to as *expression*

profiling, is perhaps the most common microarray application.

A *microarray* is typically a glass slide, silicon wafer, or even a plastic substrate upon which very large numbers (currently hundreds of thousands) of portions of individual gene sequences (genomic or cDNA) have been permanently applied [18, 19]. These devices are sometimes referred to as *gene chips*. It is worth noting that other microarray-based technologies are currently available, such as protein microarrays [19, 20] (commonly known as *protein biochips*) and antibody (Ab) microarrays. Consequently, microarray designs fall into three categories: (1) genomic arrays, which are used to study the structure and organization of genomic DNA; (2) transcriptome arrays, which are used to measure gene expression at the level of RNA synthesis; and (3) proteomic arrays, which are used to measure protein expression and also to study protein interactions.

Originally, each microarray was printed with sequences representing a unique tissue. Today, however, multiple-tissue microarrays are becoming increasingly popular, thereby facilitating the simultaneous assay of several tissues. This approach is analogous to the very popular multiple-tissue Northern blots that are available commercially from many biotech suppliers, where RNA from several tissues has been blotted and is ready for nucleic acid hybridization. In a way, multiple-tissue microarrays represent a high-tech, high-throughput extension of *in situ* hybridization, in which gene expression is assigned to specific cell types within the architecture of a tissue sample. Microarrays are also available with various themes, such as a cancer array (sometimes referred to as a *cancer panel*). These specialized microarrays, as well as microarrays printed with broad-ranging sequences, are designed to provide investigators with as much latitude as possible in designing their experiments and interpreting the very large amount of resultant data.

Microarrays are probed in a very small volume (200 μl) of hybridization buffer overnight with labeled cDNA or cRNA from two different sources, such as a treated sample and a control sample. Each cDNA probe is labeled with a different fluorescent dye, most often Cy3 (green fluorescence) and Cy5 (red fluorescence). These probes are mixed together and used to cohybridize to targets printed on the microarray (Fig. 7). If Cy3-labeled cDNA and Cy5-labeled cDNA hybridize to the same spot on a microarray, the laser-induced fluorescence of both Cy3 and Cy5 makes the spot appear yellow. If either probe alone hybridizes to a sequence on the array, then green or red fluorescence will be observed, which means that the corresponding gene is expressed in one or the other sample alone. Thus, it is the fluorescence ratio-based analysis of each spot on the microarray that provides information concerning the abundance of particular transcripts. Although microarrays are considered high-throughput tools, they are not especially quantitative. Candidate genes are generally identified by high-throughput microarray analysis, after which the behavior of the genes may be more fully characterized, at least at the transcriptional level, by very quantitative real-time PCR.

Although microarray analysis is perhaps the best known format for high-throughput analysis, other methods are available in the repertoire of the molecular biologist. Among these are included the serial analysis of gene expression (SAGE) [21], mRNA differential display [22], amplified fragment length polymorphisms

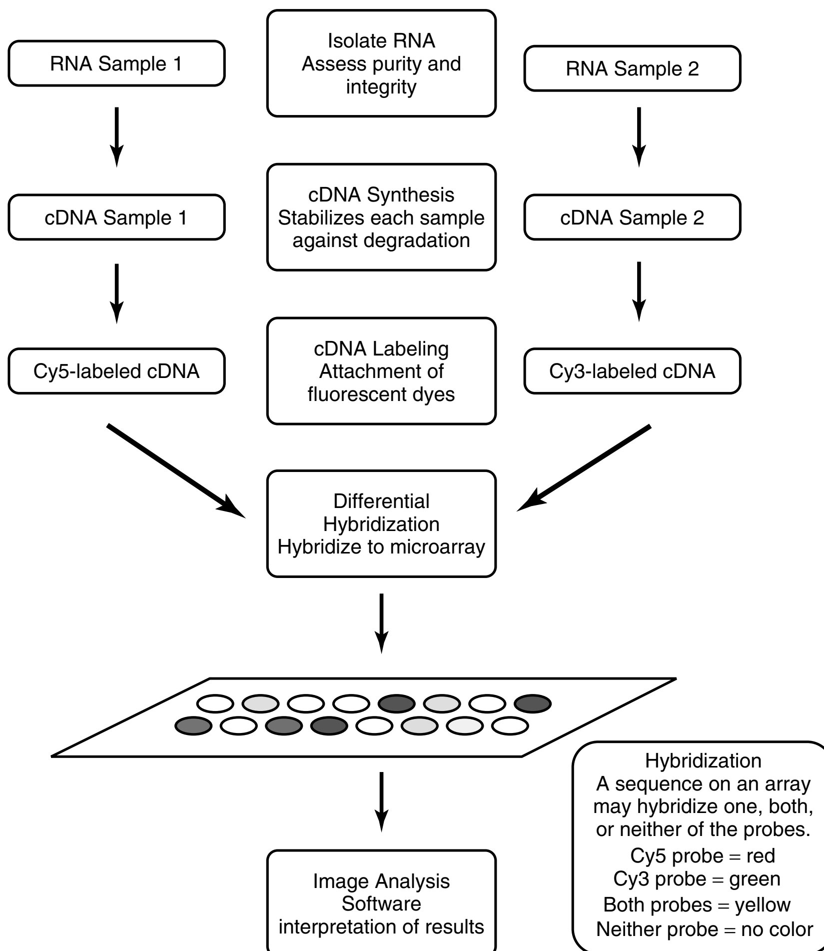

Fig. 7 Major steps in microarray analysis. cDNAs are synthesized, labeled, and hybridized to an array. Fluorescence detection coupled with image analysis provides a great deal of information of about patterns of gene expression in the samples under investigation. Reproduced with permission from Farrell Jr, R.E. (2010) *RNA Methodologies: A Laboratory Guide for Isolation and Characterization*, 4th edn, Elsevier, Academic Press, San Diego, CA, p. 503) [1].

(AFLPs) [23], massively parallel signature sequencing (MPSS) [24] and, most recently, whole-transcriptome deep sequencing (RNA-Seq) [25]. Each of these methods profiles the expression of most (or all) genes simultaneously, without prior knowledge of the identity of those genes. Consequently, any gene can be assayed without knowing ahead of time which genes should be examined, which is in direct contrast to all forms of classical PCR, in which gene-specific primers are used to target only one cDNA species for amplification. Inasmuch as these newer, highly sensitive methods are describes in detail elsewhere in this volume, the methods are briefly mentioned here as a point of reference for additional information.

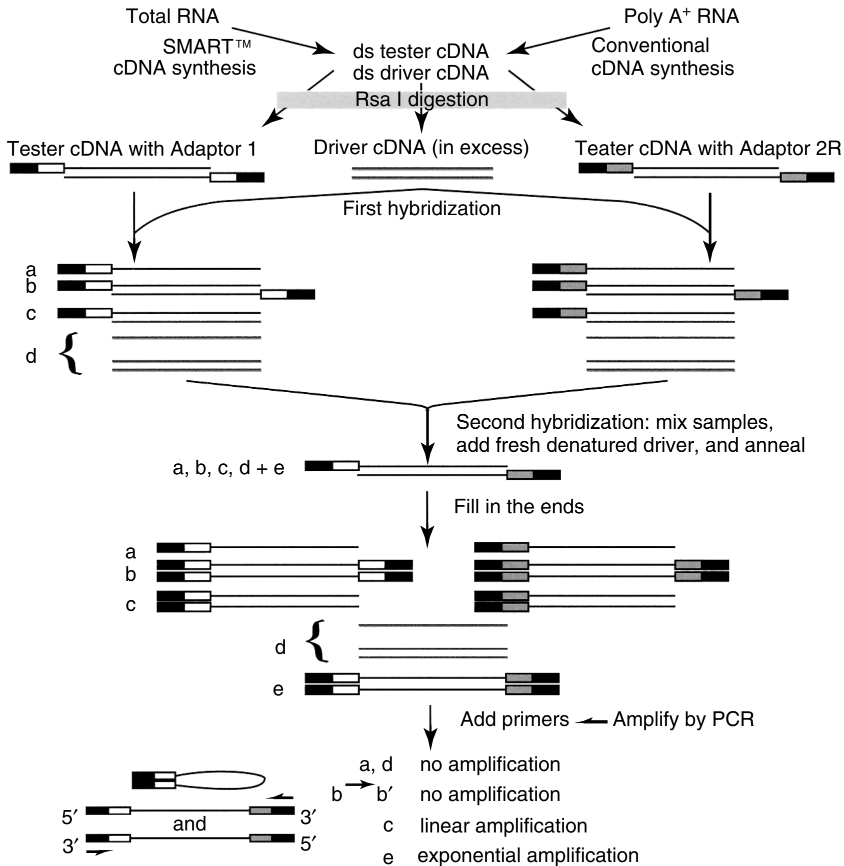

Fig. 8 Suppression subtractive hybridization. In this procedure, the tester and driver cDNA strands are able to interact with each other in various combinations, leading to enrichment of upregulated gene sequences found in the tester cDNA population. Illustration courtesy of Clontech Laboratories (PCR-Select cDNA Subtraction System).

6.7 Suppression Subtractive Hybridization (SSH)

The global identification of differentially expressed genes is also possible in a non-microarray format, and without the bioinformatics challenges associated with RNA-Seq and similar next-generation sequencing-based methodologies. In particular, suppression subtractive hybridization (SSH) is a method of nucleic acid subtraction of sequences common to control and experimental cDNA populations, coupled with the PCR amplification of uniquely expressed sequences [26, 27]. Moreover, the mechanics of the assay (Fig. 8) favor a normalization of differential expressed sequences, which means that the assay produces an enriched pool of differentially expressed sequences that are all present at a similar concentration, regardless of their respective abundance levels in the original biological material. This situation is highly desirable during

the identification process because, in a non-normalized library, highly abundant cDNAs are much more likely to be cloned compared to cDNAs of lesser abundance. Thus, the sequencing of 100 clones from a normalized cDNA library will provide a much more comprehensive, representative biochemical ''snapshot'' of the variety of up- or down-regulated sequences, compared to a non-normalized library.

In the present author's laboratory, the SSH method has been used in several applications, and has provided outstanding results [28, 29], generating a more complete profile of gene expression than did the older method of mRNA differential display. It might be expected that any sequence which differs in abundance by fivefold would be easily sequestered by the subtraction process, but it is not uncommon to detect genes with as little as a 1.5-fold difference compared to the control population.

Briefly, SSH involves creating two tester (cDNA from experimental cells) subpopulations ligated to different adapters, while no adapters are ligated to the driver (cDNA from the control cells). Subsequently, two hybridizations are performed to remove those cDNAs common to the cDNA tester and driver populations, leaving only uniquely expressed cDNAs available for amplification by PCR; the resulting PCR products are the differentially expressed genes. The enriched, differentially expressed sequences are now ready for cloning, confirmation of differential status, and sequencing. On completion of the subtraction-suppression PCR procedures, the subtracted cDNAs are ligated to plasmids, followed by transformation into *Escherichia coli*. The individual clones can be plasmid-prepped and, after sequencing, identified using various bioinformatics tools (e.g., BLAST™ analysis) that are available to the contemporary molecular biologist. Clearly, SSH represents a versatile, low-cost alternative to microarray technology.

6.8 RNAi

A remarkable new tool has emerged that is able to suppress the expression of specific endogenous genes through the use of double-stranded RNA (dsRNA). During the few short years since the first demonstration of the power of this technique in *Caenorhabditis elegans* [30], and subsequently in human and other mammalian cells [31, 32], refinements in the methodology have had the same level of impact on research that PCR did 25 years earlier. This new and emerging technology is known as RNA interference (RNAi); it is also referred to occasionally as ''gene knockdown'' or ''post-transcriptional gene silencing.'' It should be noted that RNAi is a patented process, and the commercial use of this process may require licensing through the Carnegie Institute of Washington (www.ciw.edu).

RNAi is a natural phenomenon that was considered an oddity when it was first observed in petunias [33, 34]. It is now known to exist in many organisms as a means of protecting against viruses and other molecular invaders that would otherwise plague and wreak havoc with a host genome. This method of protecting the integrity of the eukaryotic genome is highly conserved, and involves the targeting of dsRNA entering the cell for its immediate destruction. Natural processes mediated by RNAi include the turnover of wild-type and mutant mRNAs, translational regulation during the development of an organism and, undoubtedly, also

other regulatory mechanisms in the cell that have not yet come to light.

RNAi is an endogenous catalytic pathway that is triggered by dsRNA. The "trigger" can occur either naturally, as in the case of a cellular infection by a dsRNA virus, or by the intentional introduction of dsRNA to induce a user-directed degradation of the complementary transcript(s). The net result of RNAi is the downregulation of specific genes by the destruction of their mRNA(s). This method of studying the effects of gene expression in a cell or in an organism is loosely referred to as *reverse genetics*, the goal of which is to determine the consequences for a cell or an organism when a protein is *not* produced. In addition to developmental biology, RNAi has profound ramifications in the treatment of infectious diseases, and in other diseases that result from inappropriate protein expression, such as gain-of-function mutations. The major strength of RNAi is that it permits the study of the function of one gene at a time over an extended period.

Recently, RNAi has emerged as an important mainstream tool for both basic and applied research, and has already revolutionized the area of study known as *functional genomics*. RNAi is ubiquitous in eukaryotes, and currently is a favored tool for investigating the regulation of gene expression in plants, animals, and fungi. This technology is becoming increasingly popular owing to its compatibility with cell culture as well as *in vivo* research models. Comparatively speaking, the process of RNAi is much faster and far more economical than creating knock-out animals in order to study the function of specific genes. The resulting precision silencing of specific genes also makes RNAi an attractive platform for the discovery and development of life-saving pharmaceuticals.

There are multiple approaches by which RNAi can be induced, each of which has several mechanistic permutations. RNAi is, fundamentally, a two-step process (Fig. 9). The first step involves one of the master enzymes in the RNAi process, a type III endoribonuclease aptly named Dicer. This enzyme, a ubiquitous member of the eukaryotic proteome, is involved in the ATP-dependent cleavage

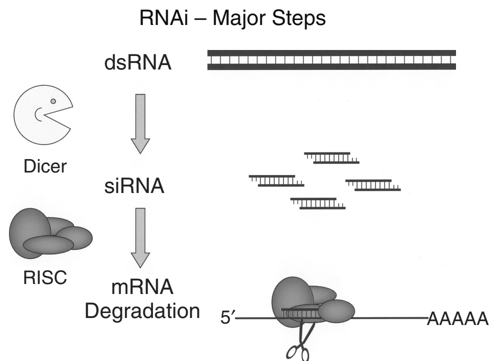

Fig. 9 Major steps in the RNAi process. Double-stranded RNA (dsRNA) from any of a number of sources, and in any of a number of configurations, is cut by the enzyme Dicer into siRNA which, in turn, become part of the multicomponent RISC. This ultimately leads to destruction of the target mRNA and concomitant downregulation of the associated gene. Reproduced with permission from Farrell Jr, R.E. (2005) *RNA Methodologies: A Laboratory Guide for Isolation and Characterization*, 3rd edn, Elsevier, San Diego, CA, p. 607) [1].

of long dsRNA into 21–23 bp short inhibitory RNA (siRNA) molecules with characteristic 3′ dinucleotide overhangs on both strands [35]. Long dsRNA can be introduced into mammalian cells, where it will become a substrate for Dicer, although care must be taken to ensure that the dsRNA is not so long as to induce the interferon pathway, leading to apoptosis. A more prudent choice is to use recombinant Dicer to generate siRNA *in vitro*, after which these short molecules are introduced into the cell by transfection. In the second step, siRNA, regardless of the source, becomes part of a multicomponent nuclease-containing RNA-induced silencing complex (RISC). Part of the RISC is an ATP-dependent helicase that unwinds the double-stranded siRNA, the antisense component of which is now able to base-pair with the mRNA to be silenced. The formation of a double-stranded region between the antisense component of the silencing RNA and the cognate mRNA seals its fate. The RISC cuts the mRNA close to the middle of this transiently formed double-stranded region [36], after which the mRNA is further degraded, preventing any level of interaction with the cellular translation machinery.

It is worth noting that there remains much confusion as to the functional similarities and differences between siRNA and miRNA. In terms of the net result, miRNA and siRNA actions are often indistinguishable; hence, the major difference is not what these molecules do, but rather where they come from. miRNAs result from the endogenous transcription of genomic DNA; eukaryotic cells are also replete with the nuclear enzyme *Drosha*, which is responsible for the initial post-transcriptional processing of naturally occurring nuclear transcripts that will mature into miRNA molecules. In contrast, siRNAs are the result of either the processing of long dsRNA, the processing of short hairpin RNA (shRNA) molecules that are produced by expression vector transcription, or by the direct introduction of siRNA via transfection. Both, siRNAs and miRNAs, mature through the action of one of the cytoplasmic *Dicer* enzymes, which leads to their association with a RISC-like complex. The major functions of miRNA are translational repression and mRNA cleavage, while siRNAs tend to be associated with mRNA cleavage alone. The subtleties of RNAi are described in much greater detail elsewhere in the EMCBMM.

6.9 *In Vitro* Translation

The classical methods used for the analysis of RNA include all of the standard techniques described above, and many others. Another aspect of the RNA story, however, is the destiny of those transcripts which emerge as mature mRNA molecules in the cytoplasm. mRNA biogenesis guarantees neither translation nor translatability, and a gene is really not ‘‘expressed’’ until a functional peptide is produced. In order to more fully characterize gene expression, the extent of translation may be assessed by Western analysis [37], and the resulting data correlated to the transcriptional activity of the corresponding gene(s).

The translation of mRNA into protein *in vitro* goes hand-in-hand with the quantification of that transcript as a parameter of gene expression. Briefly, mRNA synthesized naturally or by *in vitro* transcription is added to a whole-cell lysate containing the components needed to support the *in vitro* translation of those mRNAs. The translation is then performed in the presence of radiolabeled amino acids or,

more recently, of amino acid labels that support nonisotopic detection. Common *in vitro* translation systems include the reticulocyte lysate system and the wheat germ extract system, and in some cases, the microinjection of message directly into a living cell. The pretreatment of an *in vitro* translation lysate with micrococcal nuclease destroys endogenous mRNA, ensuring *de novo* protein synthesis exclusively from the experimental mRNA added to the system. The same *in vivo* requirements for 5′ cap, initiation codon, and polyadenylation apply here if the experimental introduced message is to be translated efficiently. The protein products are then analyzed as usual by using Western analysis, biological assay, immunopurification, or related techniques. This particular aspect of biotechnology is attractive for a number of different applications, including the study of naturally occurring animal and plant mRNAs, transfection experiments, the characterization of mRNA products, protein engineering, the screening and analysis of mutants, and DNA-, RNA-, or protein-binding studies.

7 Summary

The characterization of RNA from biological sources is a central action of profound significance in molecular biology. Gene expression is frequently analyzed using blot analysis, by solution hybridization methods, or by any of a number of RT-PCR variations. Increasingly, many of the classical RNA analysis methodologies have become sidelined or even passed over altogether because of the power of PCR, and the fact that the methods described here have revolutionized many aspects of biotechnology is not in doubt. RNA is analyzed to answer transcription questions, and the tools described in this chapter are currently in widespread use to this end. The method by which RNA is isolated, the downstream technique(s) used to analyze the RNA, and the level of sensitivity associated with that technique are all variables that must be weighed up before any laboratory investigations are undertaken. RNA methodologies have a non-negotiable requirement for high-quality starting materials if the ensuing data are to be representative, to accurately reflect the cellular biochemistry, and to provide insight into the subtleties of gene regulation.

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