Swayam Course - Analytical Techniques

Week 11, Tutorial 27 - Methods to Quantify and Integrity check of RNA

Content Writer - Dr. Rakhee Yadav, Assistant Professor, Department of Biochemistry, AIIMS, New Delhi

INTRODUCTION

The isolation/extraction of RNA from cells followed by the analysis with techniques such as RNA sequencing (RNA-seq) or microarrays tells about the functional state of the cells or tissues. It also gives an insight as to how the cells respond to a specific stress or stimulus.

Extracted RNAs can tell us which genes are expressed or transcribed in which relative abundance i.e. what is the expression profile of genes looks like and to which degree they are active.For example, to understand in which way specific cells responded upon treatment by a drug, how cancers respond upon oxygen starvation, or lung tissue upon infection with a virus.

We all know that DNA is the storehouse of genetic material which includes all the protein-coding genes. It involves the regulatory sequences, euchromatin and heterochromatin regions. During transcription, DNA is transcribed into mRNA (messenger RNA) which then translates to form different proteins. Therefore, the RNA template is the deciding factor for all the proteins that are to be synthesised and that's why the extraction of RNA and its analysis has following applications:

- Northern Blotting: A method to determine the abundance and size of an RNA molecule in a sample, by hybridization with a gene specific probe
- RT-PCR and Real Time PCR (qPCR) for quantification of gene expression
- Microarrays: A technique to examine the expression levels of thousands of genes ina single experiment.
- cDNA library construction which is specific to the cell type.

OBJECTIVES

- To have a brief overview of basic RNA structure & chemistry.
- To learn about the RNA isolation strategies and to discuss the problem of ribonuclease (RNase) contamination.
- To learn the principles and steps involved in organic extraction of total RNA with its protocol.
- To understand the strategies for the assessment of purity and integrity of isolated RNA

RNA STRUCTURE AND CHEMISTRY

RNA is the long unbranched polymer of ribonucleoside monophosphate joined together by pdosphodiester linkages. RNA is a single stranded molecule.

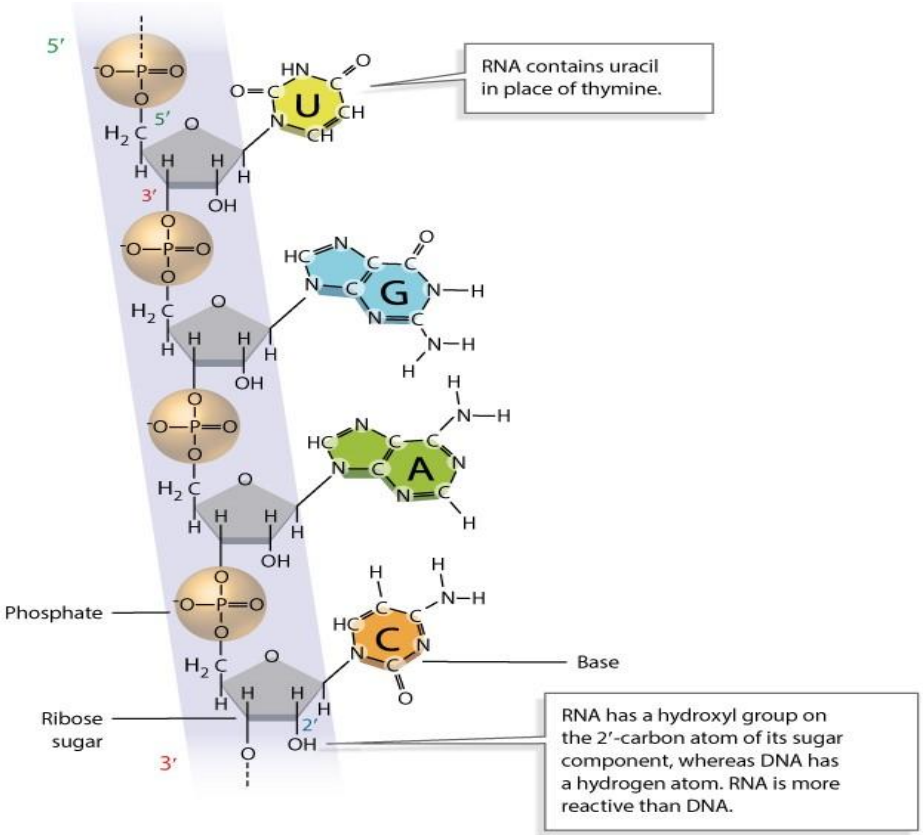

Figure 1 : Structure of RNA.
A region of single-stranded RNA. The sugar component of the sugar-phosphate backbone is ribose: a five carbon sugar with a hydroxyl group on its 2' carbon atom. The sugar component of the sugar-phosphate backbone in DNA is deoxyribose: a five carbon sugar with a hydrogen atom, instead of a hydroxyl group, on its 2' carbon atom. Additionally, RNA does not contain the nitrogenous base thymine (T), found in DNA. Instead, the nitrogenous base uracil (U), not found in DNA, binds with the base adenine

Cellular "total" RNA is actually made up of:

•Messenger RNA (mRNA): 1-5% and serves as a template for protein synthesis.

•Ribosomal RNA (rRNA): >80% and most abundant of cellular total RNA. It is the structural component of ribosomes.

•Transfer RNA (tRNA): 10-15% and translates mRNA information into the appropriate amino acid.

RNA ISOLATION STRATEGIES:

RNA is chemically and biologically more labile than DNA especially in presence of alkali and at elevated temperatures (>65°C). This is attributed to its 2'-OH group and single stranded structure (figure 1). The stability of RNA is further reduced by the presence of variety of ribonucleases (RNase), which are ubiquitous in nature. Hence the isolation of RNA becomes challenging. Thus, RNA must be isolated rapidly with the use of reagents free from RNase contamination

Isolation of RNA begins with cellular lysis mediated by buffers that fall into one of the following two categories:

a. Harsh chaotropic agents like Guanidium salts, SDS, Urea which lyse the plasma membrane and subcellular organelles and simultaneously inactivates RNase.

b. Non-ionic, hypotonic lysis buffers e.g. NP-40 which gently solubilises the plasma membrane and maintains the structural integrity of subcellular organelles.

1. **Method of membrane solubilisation:**

The very first factor in the design of RNA isolation strategy is the method of cellular disruption, which is based on the population of RNA or subcellular compartment the investigator wishes to study. For example harsh detergents solubilise all membranes and release total RNA into the solution which can be analyzed. This would be suitable to study the levels of a particular RNA inside a cell irrespective of cellular localization. However to study the cytoplasmic levels of a particular RNA, the cell membrane will be solubilised with a mild detergent leaving the nuclei intact. When RNA is isolated now, we will obtain only the cytoplasmic RNA which gives us the organelle specific RNA levels.

Table 1: Populations of RNA in cell

mRNA subpopulations	**Composition**
Total cellular RNA	All RNA from the cell
Total cytoplasmic RNA	Cytoplasmic RNA excluding mitochondrial RNA
Nuclear RNA	Transcripts (mature and immature) isolated directly from intact nuclei
mRNA	Transcripts that have matured and been exported to the cytoplasm. This subpopulation includes both poly A+ and poly A- transcripts
Cellular poly $(A)^+$ RNA	Nuclear & cytoplasmic polyadenylated transcripts
Cytoplasmic poly $(A)^-$ RNA	Cytoplasmic polyadenylated mRNA
Poly $(A)^-$ RNA	Non- polyadenylated RNA mostly rRNA and tRNA
mtRNA	Mitochondrial RNA (all types)

2. **Total inhibition of RNase (ribonuclease) activity must be ensured:**

RNase are a family of enzymes, sequestered intracellularly in virtually all living cells. They are liberated upon cell lysis and can degrade RNA. Extrinsic sources of RNase include bottles and containers in which chemicals are packaged, water, gel boxes and combs, microbial and fungal contamination of buffers. The single greatest source of contamination is oil fromour fingertips, which is rich in RNase activity.

RNases have minimal co-factor requirement and are active over a wide pH range. RNases especially those belonging to the RNaseA family, are fairly small, compact proteins containing several cysteine residues that form numerous intramolecular disulfide bonds. As a result, denatured RNases tend to regain their native structure and partial function after being cooled to room temperature in the absence of a denaturant. Consequently, RNases can retain substantial activity after freeze-thaw cycles and even autoclaving. The robust nature of these enzymes makes them refractory to many methods of decontamination, and strong chemical methods are often required to eliminate RNases from surfaces and solutions.

So, proper measures need to be taken:

- ✓ Wear gloves during experiments to prevent contamination from RNases found on human hands.
- ✓ Change gloves after touching skin (e.g., your face), doorknobs, and common surfaces.
- ✓ Have a dedicated set of pipettes that are used solely for RNA work.
- ✓ Use tips and tubes that are tested and guaranteed to be RNase-free.
- ✓ Use RNase-free chemicals and reagents.
- ✓ Designate as an "RNase-free zone" a low-traffic area of the lab that is away or shielded from air vents or open windows.
- ✓ Include a chaotropic agent (Guanidine) in the procedure which can inactivate andprecipitate RNases and other proteins.

- ✓ All the consumables can be pre-treated with chemicals like DEPC (Diethyl pyrocarbonate) to remove RNase activity.

3. **Method for deproteinisation of the sample:**
   The complete removal of proteins from the cell lysate is of paramount importance, as the carry over proteins can inhibit reverse transcription and amplification by PCR. Removal of proteins can be accomplished by:
   - ✓ Digestion of the sample with the enzyme proteinase K.
   - ✓ Repeated extraction with mixtures of organic solvents such as phenol and chloroform.
   - ✓ Solubilisation in Guanidinium buffers.
   - ✓ Physical separation by (silica) column chromatography.
   - ✓ Salting-out of proteins.
   - ✓ Any combination of the above
4. **Nucleic Acid concentration:**
   This is generally the final step in the isolation of RNA. The most common method used for concentrating nucleic acid is precipitation using various combinations of salt and alcohol. The addition of salt allows the RNA molecules to come together instead of repelling each other and also reduces the hydrophilic nature of RNA thus making it easier for RNA to precipitate out of solution when alcohol is added. The use of the newer silica-based technologies often precludes the need to add any salt or alcohol because the RNA can be eluted from the column in concentrated form and the yield is also higher as compared to alcohol based procedures.
5. **Selection of proper storage conditions for purified samples of RNA**
   Because of the labile nature of RNA, improper storage often leads to degradation in relatively short time. For proper storage of RNA the appropriate temperature, buffer and storage form need to be considered. Purified RNA is most stable when stored as ethanol precipitate at -80°C. RNA can be store for several months under these conditions. If the sample is needed to be used within weeks it can be stored as ethanol precipitate at -20°C. The RNA degradation is accelerated when RNA is dissolved in aqueous buffer either water or TE buffer.

ORGANIC EXTRACTION OF TOTAL RNA: ACID GUANIDINIUM- PHENOL-CHLOROFORM EXTRACTION TECHNIQUE (TRIZOL AGENT)

High quality RNA can be extracted using chaotropic lysis buffer and Guanidium containing buffers are among the most effective. In this method the RNA is isolated in very short time by the usage of a guanidium thiocyanate with an acidic phenol solution. Chloroform added to facilitate partitioning of the aqueous and organic phase. Upon phase separation, RNA is retained in the aqueous phase, while the DNA and proteins partition into the organic phase. RNA is then recovered by precipitation with isopropanol and collected by centrifugation (Figure 2).

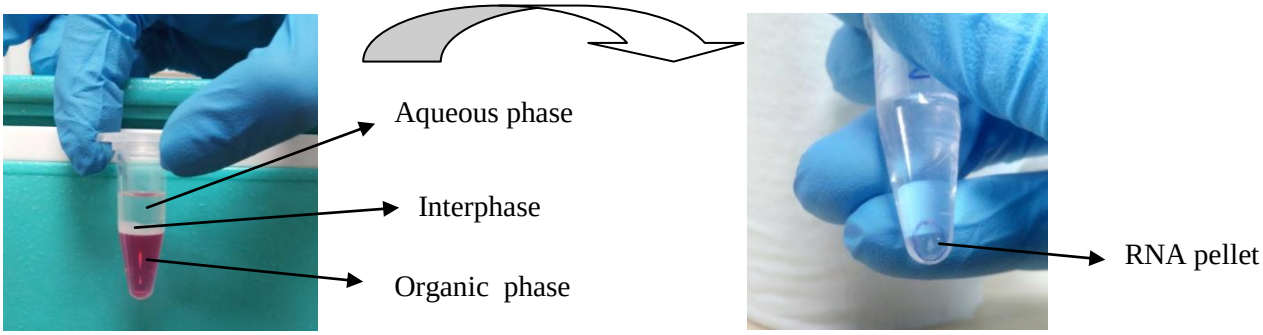

Figure 2: Representation of the phase separation in phenol-chloroform extraction of RNA (Image source: content provider's own lab)

Prototcol of TRIzol method of RNA isolation:

I. **Sample Preparation:**

i) **Tissue:** Homogenize tissue samples in TRI Reagent (1 ml per 50–100 mg of tissue) in an appropriate homogenizer. The volume of the tissue should not exceed 10% of the volume of the TRI Reagent.

ii) **Monolayer cells:** Lyse cells directly on the culture dish. Use 1 ml of the TRI Reagent per 10 cm^2 of culture plate surface area. After addition of the reagent, the cell lysate should be passed several times through a pipette to form a homogenous lysate.

iii) **Suspension cells**: Isolate cells by centrifugation and then lyse in TRI Reagent by repeated pipetting. One ml of the reagent is sufficient to lyse 5–10 x 10^6 animal, plant, or yeast cells, or 10^7 bacterial cells.

II. **Phase Separation**: To ensure complete dissociation of nucleoprotein complexes, allow samples to stand for 5 minutes at room temperature. Add 0.2 ml of chloroform per ml of TRI Reagent used. Cover the sample tightly, shake vigorously for 15 seconds, and allow to stand for 2–15 minutes at room temperature. Centrifuge the resulting mixture at 12,000 g for 15 minutes at 2-8 °C. Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA).

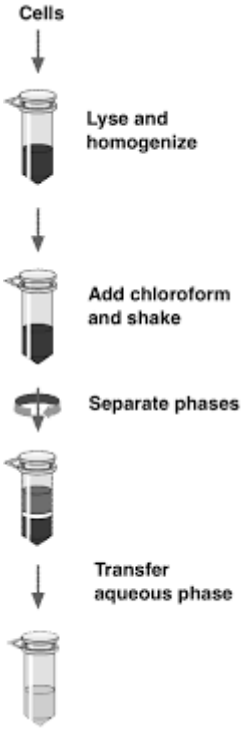

Figure 3: An overview of steps of total RNA extraction
(http://physiology.med.cornell.edu/faculty/mason/lab/zumbo/files/PHENOL-CHLOROFORM.pdf)

The separation of different molecules in three phases is the heart of this method. Following explanations are useful in understanding the biochemical basis of this phenomenon:

- For RNA extraction the typical ratio of phenol to chloroform is 5:1(v/v). At acidic pH, this ratio results in the absence of DNA from the upper aqueous phase.
- Guanidium salts are used to reduce the effects of nucleases.
- Purified phenol has a density of 1.07g/cm^3 and therefore forms the lower phase when mixed with water (1.00g/cm^3). Chloroform ensures phase separation of the two liquids because chloroform is miscible with phenol and it has a higher density (1.47g/cm^3) than phenol; it forces a sharper separation of the organic and aqueous phases thereby assisting in the removal of the aqueous phase with minimal cross contamination from the organic phase (figure 3).
- Nucleic acids are polar because of their negatively charged phosphate backbone, and therefore nucleic acids are soluble in the upper aqueous phase instead of the lower organic phase (water is more polar than phenol).

- On the other hand; proteins contain varying proportions of charged and uncharged domains, producing hydrophilic and hydrophobic regions. In the presence of phenol; the hydrophobic cores interact with phenol, causing precipitation of proteins and polymers to collect at the interface between the two phases.
- Lipids to dissolve in the lower organic phase.
- ***Effect of pH:*** Acid phenol specifically leaves RNA in the aqueous phase. As the pH decreases, the concentration of protons increases. DNA carries a negative charge due to the phosphate groups in its sugar-phosphate backbone, which are neutralised in acid by protonation. In this case, therefore, DNA dissolves in the organic phase (like dissolves like). RNA, on the other hand, is not neutralised in acid because even though it also has a negative charge, it has exposed nitrogenous bases (being single-stranded), which can form hydrogen bonds with water, thereby, keeping it in aqueous phase.

III. **RNA Isolation**:

i) Transfer the aqueous phase to a fresh tube and add 0.5 ml of isopropyl alcohol per ml of TRI Reagent used in Sample Preparation and mix. Allow the sample to stand for 5-10 minutes at room temperature. Centrifuge at 12000g for 10 minutes at 2-8 °C. The RNA precipitate will form a pellet on the side and bottom of the tube.

ii) Remove the supernatant and wash the RNA pellet by adding a minimum of 1 ml of 75% ethanol per 1 ml of TRI Reagent used in Sample Preparation. Vortex the sample and then centrifuge at 7,500 g for 5 minutes at 2-8 °C.

iii) Briefly dry the RNA pellet for 5–10 minutes by air drying. Do not let the RNA pellet dry completely because this will greatly decrease its solubility.

The total RNA isolation by phenol-chloroform based organic extraction is versatile and is compatible with a variety of sample types. It has an advantage of being established and proven technology which is inexpensive too. But use of organic solvents and more chances of contamination with genomic DNA are its major disadvantages; therefore some techniques which promise excellent RNA purity and integrity are:

FILTER BASED SPIN BASKET FORMATS

They utilise membranes (usually glass fibre, derivatised silica, or ion exchange membranes) that are seated at the bottom of a small plastic basket. Samples are lysed in a buffer that contains RNase inhibitors (like Guanidium salts), and nucleic acids gets bound to the membrane on passing the lysate through the membrane using centrifugal force. Wash solutions are subsequently passed through the membrane and discarded. An appropriate elution solution is applied and the sample is collected into a tube by centrifugation.

Such techniques have the advantages that they eliminate the need for organic solvents and yield RNA which is of excellent purity and integrity.

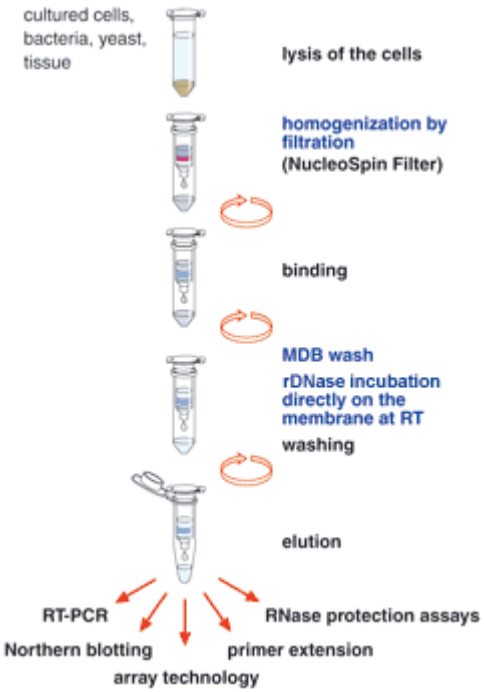

Figure 4: Filter based, spin basket format for RNA extraction

MAGNETIC PARTICLE METHODS

These methods utilise small (0.5-1µm) particles that contain a paramagnetic core and surrounding shell modified to bind to entities of interest. Paramagnetic particles migrate when exposed to a magnetic field. This allows the particles to interact with molecules of interest based on their surface modifications, be collected rapidly using an external magnetic field, and then be resuspended easily once the field is removed. Samples are lysed in a solution containing RNase inhibitors and allowed to bind to magnetic particles. The magnetic particles and associated cargo are collected by applying a magnetic field. Finally RNA is released into the elution solution.

mRNA ISOLATION:

mRNA molecules have a polyA tail at the 3’ end (poly A tail). Oligo(dT) probes can be used to purify mRNA from other RNAs. mRNA can then be eluted from oligo(dT) matrix using water or low salt buffer in a column chromatography or using oligo (dT) coupled magnetic beads in a magnetic separation method

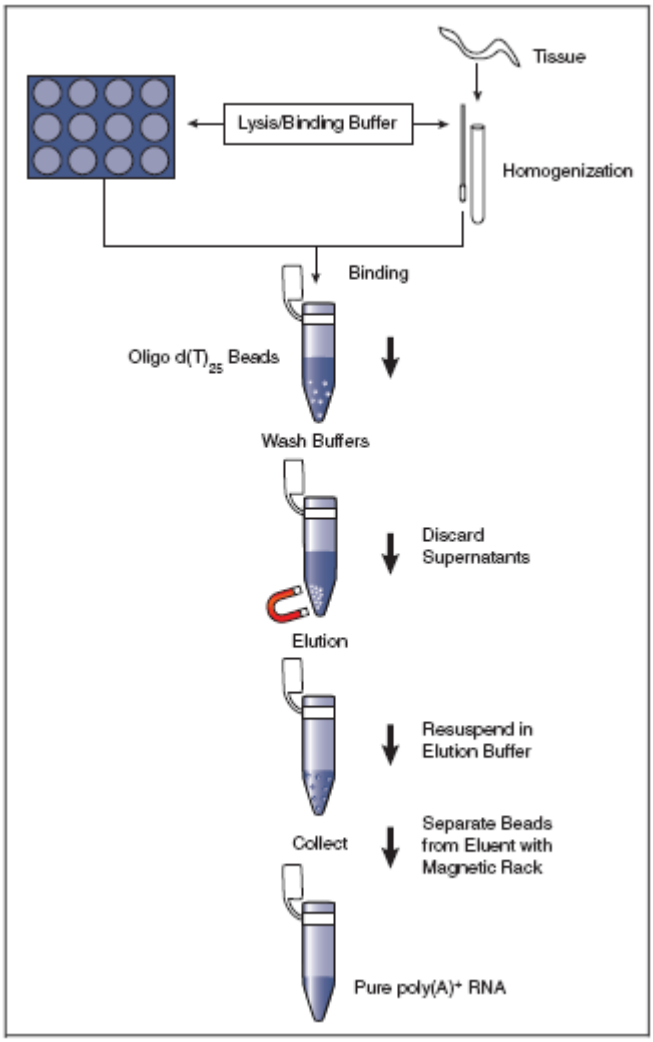

Figure 5: mRNA isolation using magnetic oligo(dT) beads

RNA QUALITY ASSESSMENT

Once we have got the RNA extracted, we need to check for its Ppurity" and "Integrity". By Purity; we mean that the RNA sample must be free from contaminants like proteins and other organic compounds. In addition to being pure; extracted RNA must also be intact i.e. not degraded.

Let us now have an overview of the methods for RNA quality assessment.

1) Test for RNA purity:

Spectrophotometric method:

Nucleic acids show maximum absorbance at 260nm. So, the absorbance of the extracted RNA sample is determined on the spectrophotometer including any NanoDrop spectrophotometer at 260nm, 280nm and 230nm. This is because following molecules show maximum UV absorbance at such wavelengths:

260nm: Nucleic acids (DNA, RNA, nucleotides)

280nm: Proteins

230nm: Guanidine

The ratio of A_{260}/A_{280}: Pure RNA will exhibit this ratio in the range of 1.8-2.0. If this ratio is lower than 1.7 then it indicates protein contamination in the sample.

Further**, Ratio of A_{260}/A_{230}:** Purified RNA should exhibit this ratio within range of 2.0-2.2 and it indicates presence of contaminants like guanidine in the sample if the ratio is lower.

RNA quantitation: For RNA; 1OD_{260}Unit=40µg/ml of ssRNA. The concentration in the sample is calculated by using the formula: A_{260} x dilution x 40 = [RNA]µg/ml.

2) **Tests for RNA integrity:**

Gel electrophoresis

The RNA extracted using various isolation techniques needs to be checked for its quality because of labile nature of RNA. This can be done by electrophoresis of native RNA in 2% agarose gel. Like DNA; RNA is also separated on electrophoresis on the basis of its size. RNA contains some secondary structures into which Ethidium bromide intercalates and thus RNA can be visualized. Ribosomal RNA forms a major portion of the total RNA extracted (around 80 %) and two rRNA bands for 18S and 28S are clearly visible upon electrophoresis and staining. any RNA degradation will be seen as a smear (figure 6).

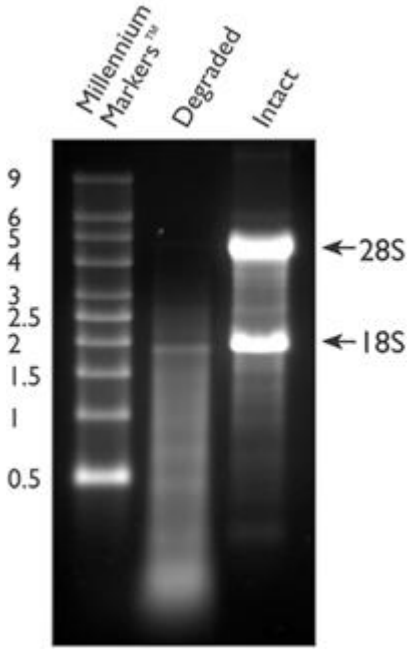

Figure 6: Comparison of degraded and intact gel electrophoresis picture of total extracted RNA sample

In order to determine the exact size of RNA; complete denaturation of RNA with chemicals like formaldehyde, glyoxal, methylmercuric acid is required. These denaturating agents are added in the agarose gel as well as in the electrophoresis buffer. After heat denaturation of RNA they form adducts with the amino group of guanine and uracil and prevent intra chain as well as intra polynucleotide hydrogen bond formation. Denatured RNA cannot be stained with ethidium bromide because of the absence of secondary structures to intercalate. Hence acridine orange which can intercalate as well as bind with polynucleotides electrostatically is used for staining of denatured RNA. Apart from determining the size of RNA, denaturing gel electrophoresis is a preparative process before which RNA can be transferred to a nylon membrane and a Northern blot performed.

RNA integrity number (RIN)

The RIN is an algorithm for assessing integrity values to RNA measurements. This algorithm is applied to electrophoretic RNA measurements, typically obtained using capillary gel electrophoresis, and based on a combination of different features that contribute information about the RNA integrity to provide a more universal measure. The algorithm assigns 1 to 10 RIN score, where level 10 is completely intact.

SUMMARY

- Extracted RNAs can tell us which genes are expressed or transcribed in which relative abundance

- RNA extraction is an essential pre-requisite for a number of techniques in diagnostics and research like real time PCR, Northern blotting, RNA-seq/Microarrays and cDNA library construction.
- Extraction of RNA is challenging as compared to DNA; owing to its unstable nature which is attributed to it by a reactive free 2'-OH group, single stranded configuration and susceptibility to RNases
- RNases are ubiquitous and robust enzymes which degrade RNA promptly
- Inhibition of RNases is most crucial to get intact RNA. A number of steps are taken to ensure this.
- Total RNA extraction is the most widely used strategy and Acid-Guanidium phenol-chloroform extraction technique is used for this. Acidic medium and organic solvents facilitate the separation of RNA, DNA and proteins in different phases of the sample. RNA comes in the aqueous phase. Guanidium causes inhibition of RNases
- Techniques like spin baskets or magnetic beads produce RNA of good yield and purity. To get mRNA fraction oligo(dT) probes are used.
- Extracted RNA is checked for its purity using spectrophotometric measurements. We can also get to know the concentration of RNA (yield) in the sample.
- To check for the integrity of RNA; gel electrophoresis is done for the extracted RNA samples and distinct bands against 28S and 18S suggest that the RNA is intact and not degraded. A smeared pattern is suggestive of poor quality of RNA extracted which has been degraded during the process of isolation; may be by RNases.
- The RIN (RNA integrity number) is an algorithm for assessing integrity values to RNA measurements. This algorithm is applied to electrophoretic RNA measurements, typically obtained using capillary gel electrophoresis. The algorithm assigns 1 to 10 RIN score, where level 10 is completely intact.