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Extraction, Purification, and Analysis of mRNA from Eukaryotic Cells

A typical mammalian cell contains about 10^{-5} μg of RNA, 80–85% of which is ribosomal (chiefly, 28S, 18S, and 5S), whereas 10–15% is made up of a variety of low-molecular-weight species (transfer RNAs, small nuclear RNAs, etc.). All of these RNAs are of defined size and sequence and can be isolated in virtually pure form by gel electrophoresis, density gradient centrifugation, or ion-exchange chromatography. By contrast, mRNA, which makes up between 1% and 5% of the total cellular RNA, is heterogeneous both in size (from a couple of hundred bases to several kilobases in length) and sequence. However, virtually all mammalian mRNAs carry at their 3′ ends a poly(A) tract that is generally long enough to allow mRNAs to be purified by affinity chromatography on oligo(dT) cellulose. The resulting, physically heterogeneous population of molecules collectively encodes all of the polypeptides synthesized by the cell.

The keys to obtaining good preparations of eukaryotic mRNA are to minimize ribonuclease activity during the initial stages of extraction and to avoid the accidental introduction of trace amounts of ribonuclease from the glassware and solutions. The following four elements are therefore important in the successful isolation of mRNA from mammalian cells.

1. Use of Exogenous Inhibitors of RNases

Two types of inhibitors of RNase are currently in widespread use: vanadyl-ribonucleoside complexes and RNasin.

Vanadyl-ribonucleoside complexes. The complexes formed between the oxovanadium ion and any of the four ribonucleosides are transition-state analogs that bind to many RNases and inhibit their activity almost completely (Berger and Birkenmeier 1979). The four vanadyl-ribonucleoside complexes are added to intact cells and used at a concentration of 10 mM during all stages of RNA extraction and purification. The resulting mRNA is isolated in high yield and in a form that can be translated efficiently in cell-free, protein-synthesizing systems. If necessary, the vanadyl-ribonucleoside complexes can be removed from the RNA preparation by multiple extractions with phenol containing 0.1% 8-hydroxyquinoline.

Vanadyl-ribonucleoside complexes are prepared as follows (Berger and Birkenmeier 1979):

1. Dissolve 0.5 mmole of each of the four ribonucleosides in 8 ml of water in a boiling-water bath.

2. Purge the solution with nitrogen gas, while adding 1 ml of 2 M vanadyl sulfate.

3. Adjust the pH to ~6 by dropwise addition of 10 M NaOH.

4. Adjust the pH carefully to 7.0 by dropwise addition of 1 M NaOH under nitrogen gas in the water bath. As the complexes form, the solution changes color from blue to green-black.

5. Adjust the volume to 10 ml; divide the solution into small aliquots and store at −20°C under nitrogen gas. The concentration of the complex is 200 mM. Dilute 1:20 for use.

Vanadyl-ribonucleoside complexes are commercially available from Bethesda Research Laboratories, P.O. Box 577, Gaithersburg, MD 20760.

RNasin. RNasin is a protein ($M_r \simeq 40{,}000$), isolated from rat liver and human placenta, that is a potent inhibitor of RNase. RNasin is effective during cell-free translation (Scheel and Blackburn 1979) and reverse transcription of mRNA (de Martynoff et al. 1980). Like vanadyl-ribonucleoside complexes, RNasin can be included in enzymatic reactions. Its advantage over vanadyl-ribonucleoside complexes is that it can be easily extracted with phenol.

RNasin is commercially available from a number of sources.

2. Methods That Disrupt Cells and Inactivate Nucleases Simultaneously

Proteins readily dissolve in solutions of potent chaotropic agents such as guanidinium chloride and guanidinium isothiocyanate (Cox 1968). Cellular structures disintegrate and nucleoproteins dissociate rapidly from nucleic acids as ordered secondary structure is lost. Even RNase, an enzyme that is resistant to many forms of physical abuse (such as boiling), is inactive in the presence of 4 M guanidinium isothiocyanate and reducing agents like β-mercaptoethanol (Sela et al. 1957). This combination of reagents can therefore be used to isolate intact RNA from tissues, such as the pancreas, that are rich in RNase (Chirgwin et al. 1979).

Guanidinium isothiocyanate is made up according to the following steps.

1. To a 100-g bottle of guanidinium isothiocyanate (Eastman Laboratory and Specialty Chemicals or Fluka), add 100 ml of deionized H_2O, 10.6 ml of 1 M Tris · Cl (pH 7.6), and 10.6 ml of 0.2 M EDTA. Stir overnight at room temperature.

2. Warm the solution while stirring to 60–70°C for 10 minutes to assist dissolution. Often there is a residue of insoluble material that is removed by centrifugation at 3000*g* for 10 minutes at 20°C.

3. Add 21.2 ml of 20% Sarkosyl (sodium lauryl sarkosinate) and 2.1 ml of β-mercaptoethanol to the supernatant and bring the volume to 212 ml with sterile H_2O.

4. Filter through a disposable Nalgene filter and store at 4°C in a tightly sealed, brown glass bottle.

3. Use of RNase-free Glassware and Plasticware

Sterile, disposable plasticware is essentially free of RNase and can be used for the preparation and storage of RNA without pretreatment. General laboratory glassware, however, is often a source of RNase contamination and should be treated by baking at 250°C for 4 or more hours. In addition, some workers treat glassware for 12 hours at 37°C with a solution of 0.1% diethylpyrocarbonate, which is a strong but not absolute inhibitor of RNase (Fedorcsak and Ehrenberg 1966). Before using glassware treated in this way, it is important to remove traces of diethylpyrocarbonate by heating to 100°C for 15 minutes (Kumar and Lindberg 1972) or by autoclaving. Otherwise, there is a danger that the remaining traces of diethylpyrocarbonate will inactivate the RNA by carboxymethylation.

It is a good idea to set aside items of glassware and batches of plasticware that are to be used only for experiments with RNA, to mark them distinctively, and to store them in a designated place.

A potentially major source of contamination with RNase is the hands of investigators. There is little use in going to great lengths to rid glassware of contamination if no care is taken to keep one's fingers out of harm's way. Gloves should therefore be worn at all stages during the preparation of materials and solutions used for the isolation and analysis of RNA and during all manipulations involving RNA.

4. Careful Preparation of Solutions

All solutions should be prepared using baked glassware, glass-distilled autoclaved water, and dry chemicals that are reserved for work with RNA and that are handled with baked spatulas. Wherever possible, the solutions should be treated with 0.1% diethylpyrocarbonate for at least 12 hours and autoclaved (15 minutes, liquid cycle). Note that diethylpyrocarbonate cannot be used to treat solutions containing Tris. It is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and carbon dioxide.