
Extraction and Purification of RNA

CONTROLLING RIBONUCLEASE ACTIVITY

To obtain good preparations of eukaryotic mRNA, it is necessary to minimize the activity of RNAases liberated during cell lysis by using inhibitors of RNAases or methods that disrupt cells and inactivate RNAases simultaneously as discussed below. Consequently, it is also important to avoid the accidental introduction of trace amounts of RNAase from other potential sources in the laboratory. A number of precautions that can be used to avoid problems with RNAases are listed below. Most experienced investigators do not rigorously adhere to these precautions but may employ one or more of them when difficulties are encountered. This list is intended to be used as a guide when contamination with RNAase is a problem.

Laboratory Procedures

If proper care is not taken, preparations of RNA can be contaminated with RNAases from outside sources including:

- *Glassware, plasticware, and electrophoresis tanks.* Sterile, disposable plasticware is essentially free of RNAases and can be used for the preparation and storage of RNA without pretreatment. General laboratory glassware and plasticware, however, are often contaminated with RNAases and should be treated by baking at 180°C for 8 hours or more (glassware) or by rinsing with chloroform (plasticware). Alternatively, some workers fill beakers, tubes, and other items that are to be used for the preparation of RNA with diethyl pyrocarbonate (DEPC) (0.1% in water), which is a strong, but not absolute, inhibitor of RNAases (Fedorcsak and Ehrenberg 1966). After the DEPC-filled glassware or plasticware has been allowed to stand for 2 hours at 37°C, it is rinsed several times with sterile water and then heated to 100°C for 15 minutes (Kumar and Lindberg 1972) or autoclaved for 15 minutes at 15 lb/sq. in. on liquid cycle. These treatments remove traces of DEPC that might otherwise modify purine residues in RNA by carboxymethylation.

Note: Carboxymethylated RNA is translated with very low efficiency in cell-free systems; however, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified.

Electrophoresis tanks used for electrophoresis of RNA should be cleaned with detergent solution, rinsed in water, dried with ethanol, and then filled with a solution of 3% H₂O₂. After 10 minutes at room temperature, the electrophoresis tank should be rinsed thoroughly with water that has been treated with 0.1% DEPC (see below).

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

Caution: DEPC is suspected to be a carcinogen and should be handled with care.

- *Contamination by workers.* A potentially major source of contamination with RNAase is the hands of the investigator. Disposable gloves should therefore be worn during the preparation of materials and solutions used for the isolation and analysis of RNA and during manipulations involving RNA. Because gloves remain RNAase-free only if they do not come into contact with “dirty” glassware and surfaces, it is usually necessary to change gloves frequently when working with RNA.
- *Contaminated solutions.* All solutions should be prepared using RNAase-free glassware, autoclaved water, and chemicals reserved for work with RNA that are handled with baked spatulas. Wherever possible, the solutions should be treated with 0.1% DEPC for at least 12 hours at 37°C and then heated to 100°C for 15 minutes or autoclaved for 15 minutes at 15 lb/sq. in. on liquid cycle.

Note: DEPC reacts rapidly with amines and cannot be used to treat solutions containing buffers such as Tris. Reserve a fresh, unopened bottle of Tris crystals for preparation of RNAase-free solutions.

Inhibitors of Ribonucleases

The following three types of specific inhibitors of RNAases are widely used:

- *Protein inhibitor of RNAases.* Many RNAases bind tightly ($K_i \cong 3 \times 10^{10}$) to a protein isolated from human placenta (Blackburn et al. 1977), forming equimolar, noncovalent complexes that are enzymatically inactive. In vivo, the protein is probably an inhibitor of angiogenin, an angiogenic factor whose amino acid sequence and predicted tertiary structure are similar to those of pancreatic RNAase (Kurachi et al. 1985; Strydom et al. 1985). The inhibitor, which is sold by several manufacturers under various trade names, should be stored at -20°C in 50% glycerol solutions containing 5 mM dithiothreitol. Preparations of the inhibitor that have been frozen and thawed several times or stored under oxidizing conditions should not be used; these treatments may denature the protein and release bound RNAases. The inhibitor is therefore not used when denaturing agents are used to lyse mammalian cells in the initial stages of extraction of RNA. However, it should be included when more gentle methods of lysis are used and should be present at all stages during the subsequent purification of RNA. Fresh inhibitor should be added several times during the purification procedure, since it is removed by extraction with phenol. The inhibitor requires sulfhydryl reagents for maximal activity and does not interfere with reverse transcription (de Martynoff et al. 1980) or cell-free translation of mRNA (Scheele and Blackburn 1979).
- *Vanadyl-ribonucleoside complexes.* The complexes formed between the oxovanadium IV ion and any of the four ribonucleosides are transition-state analogs that bind to many RNAases 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 a form that can be directly translated in frog oocytes

and can be used as a template in some in vitro enzymatic reactions (e.g., reverse transcription of mRNA). However, vanadyl-ribonucleoside complexes strongly inhibit translation of mRNA in cell-free systems and must be removed from the mRNA by multiple extractions with phenol (equilibrated with 0.01 M Tris·Cl [pH 7.8]) containing 0.1% hydroxyquinoline. Vanadyl-ribonucleoside complexes are available from several commercial suppliers.

- *Macaloid*. Macaloid is a clay that has been known for many years to adsorb RNAase. The clay is prepared as a slurry (see Appendix B) that is used at a final concentration of 0.015% (w/v) in buffers used to lyse cells (Favaloro et al. 1980). The clay, together with its adsorbed RNAase, is removed by centrifugation at some stage during the subsequent purification of the RNA (e.g., after extraction with phenol).

Methods That Disrupt Cells and Inactivate Ribonucleases Simultaneously

Proteins dissolve readily in solutions of potent denaturing agents such as guanidine HCl and guanidinium thiocyanate (Cox 1968). Cellular structures disintegrate and nucleoproteins dissociate from nucleic acids as protein secondary structure is lost. RNAases can recover activity after many forms of treatment (such as boiling) but are inactivated by 4 M guanidinium thiocyanate and reducing agents such as 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 RNAase (Chirgwin et al. 1979).

The protocols presented below use inhibitors of RNAase and/or methods that lead to the rapid inactivation of RNAases for the isolation of total, nuclear, and cytoplasmic RNAs from tissues and cultured cells.