Working with RNA: the basics

Avoiding, detecting, and inhibiting RNases

RNases, which play important roles in nucleic acid metabolism, are found in both prokaryotes and eukaryotes, and in practically every cell type. The human body uses RNases to defend against invading microorganisms by secreting these enzymes in fluids such as tears, saliva, mucus, and perspiration[1]. RNases are also present in flaked skin, on hair that may fall onto a bench, and in pet hair that may cling to clothing[2]. The primary source of RNases within most environments, however, is microorganisms—namely, bacteria and fungi. In this article, we discuss how to avoid, detect, and inhibit RNases when working with RNA.

About the RNase enzyme

RNases, especially those belonging to the RNase A family, are fairly small, compact proteins containing several cysteine residues that form numerous intramolecular disulfide bonds[3]. 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[4]. 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.

Precautions when working with RNA

Some basic precautions need to be taken when working with RNA. These safeguards will go a long way towards minimizing RNase contamination problems:

- 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

Avoiding sources of RNase contamination

Bodily fluids (“fingerases”)

Bodily fluids (such as perspiration and skin oils) are rich in RNase activity (we informally refer to RNases from these sources as “fingerases”). Thus, the use of ungloved hands could easily result in RNase contamination compromising critical experiments. Use gloves and make it a point to discard used gloves and wear a new pair frequently during experiments. (Tip: gloves that have contacted bare skin, touched a refrigerator handle, door handle, pipettor, pen, pencil, or laboratory phone should subsequently not be considered RNase-free.) Always wear a laboratory coat to prevent particulate materials from falling from your clothing onto your sample. Avoid working with RNA where airflow is turbulent or near surfaces that can create particulates (like a chalkboard).

Tips and tubes

High-quality laboratory plastic consumables can generally be considered RNase-free. However, tips and tubes can be an easily overlooked source of potential RNase contamination. Ensure that tips come from an unopened box and that tubes come from an unopened or carefully handled bag. Merely autoclaving will not destroy all RNase activity, since these enzymes are very robust and can regain partial activity upon cooling to room temperature[4]. Always use tips and tubes that have been tested and certified RNase-free. Thermo Fisher Scientific has a broad selection of certified RNase-free tips and tubes. When working with RNA, always use RNase-free barrier (filter) pipette tips to prevent introduction of RNases or cross-contamination of your RNA samples. Invitrogen™ RNase-free tips (including filter tips) of many sizes are compatible with most commonly used pipettors. We also supply regular and nonstick microfuge tubes, which can be used for any molecular biology experiment. Each lot of tips and tubes is rigorously tested for RNase and DNase contamination and is certified nuclease-free.

When glassware and metalware are required, treat them with Invitrogen™ RNase*Zap*™ reagent or wipes. An alternative is to bake these items, which may be more convenient if large numbers of items are involved. The baking procedure typically involves incubation in an oven at 450°F (232.2°C) for 2 hours or more (don't include tape, as it will burn). Prior to baking, be sure to wrap the metalware items and the tops of beakers and flasks with aluminum foil to prevent contamination after baking. When 2 hours is too long to wait, treating with RNaseZap reagent or wipes is a great alternative. Just make sure that the RNaseZap reagent is not in contact with surfaces of forceps, spatulas, or other reactive metalware (e.g., aluminum) for more than a few minutes, as this could lead to corrosion. Mark baked and RNase*Zap* reagent–treated items as "RNase-free" to distinguish them from untreated pieces. We recommend storing the treated equipment in a clearly marked RNase-free zone to prevent accidental contamination.

Water and buffers

Due to the ubiquitous nature of RNases, depending on the source and upkeep of equipment used to provide the water, the water and buffers used in molecular biology applications can be frequent sources of RNase contamination. Diethylpyrocarbonate (DEPC) treatment is the most common method used to inactivate RNases in water and buffers. However, certain reagents such as Tris cannot be DEPC treated. We offer a variety of Invitrogen™ buffers and water (DEPC-treated or untreated) that are subject to rigorous quality control testing and are guaranteed to be RNase-free. An alternative to DEPC treatment, Invitrogen™ RNAsecure™ reagent, is also available and can be used to treat primary amine solutions such as Tris and does not require a 2-hour treatment or autoclaving.

DEPC treatment is the most commonly used method for eliminating RNase contamination from water, buffers, and other solutions. (Note: DEPC cannot be used with certain buffers. See the paragraph below and "Alternatives to DEPC".) DEPC destroys enzymatic activity by modifying -NH, -SH, and -OH groups in RNases and other proteins. The treatment typically involves incubating the solution at room temperature with 0.1% DEPC for a few hours, usually overnight, followed by autoclaving the solution to eliminate residual DEPC. A common concern that researchers have is the sweet, "fruity" aroma detected after autoclaving DEPC-treated solutions. When DEPC breaks down during autoclaving, a small amount of ethanol is produced. The ethanol can combine with trace amounts of carboxylic acid to produce volatile esters, which give off this characteristic smell. This is not a sign of incomplete DEPC removal, and it will not interfere with any subsequent reactions.

Reagents containing primary amine groups (e.g., Tris) and some reagents containing secondary or tertiary amines (e.g., HEPES) cannot be DEPC treated. The amine groups tend to react with and "sop up" the DEPC, making it unavailable for inactivating RNases. Also, modification of the reagent's amine groups could affect its buffering capability. Solutions that cannot withstand autoclaving and thus need to be filtered, such as MOPS, also cannot be DEPC treated since autoclaving is essential for inactivating DEPC.

RNA*secure* reagent can be used as a convenient, non-carcinogenic alternative to DEPC for treating small volumes of precious reagents and solutions such as Tris and MOPS that cannot be DEPC treated. The reagent is supplied as a 25X stock. After the RNA*secure* reagent stock is diluted into the solution, the solution is heated to 60°C for 10 minutes, which "activates" the reagent. Unlike DEPC, which does not inactivate RNases introduced post-treatment, solutions treated with RNA*secure* reagent can be reheated to help eliminate new contaminants. For resuspension of RNA pellets, we also offer the Invitrogen™ RNA*secure*™ Resuspension Solution, which is supplied at a 1X working concentration.

Laboratory surfaces

Laboratory surfaces, such as benchtops, centrifuges, and electrophoresis equipment, should be assumed to be contaminated with RNases, since they are usually exposed to the environment. These surfaces get contaminated due to the presence of bacterial and fungal spores present in many laboratory environments. Likewise, dead cells shed from human skin can also lead to contamination of exposed surfaces. One should also assume that common laboratory equipment is also potentially contaminated. These surfaces can be treated with an RNase decontamination solution such as RNase*Zap* reagent, which is a combination of three different chemicals that is designed to completely inactivate RNases (and any other enzyme) immediately upon contact. Simply spray the surface with the solution and then rinse it thoroughly with nuclease-free water. RNase*Zap*™ wipes, which are towelettes presoaked with the RNase*Zap* reagent, are particularly convenient for decontaminating pipettors, benchtops, and other surfaces. Common detergent solutions are not effective and may actually exacerbate the problem, since detergents can spread the RNase contamination over a wider area.

Endogenous RNases

All tissue samples contain endogenous RNases. In lieu of immediate processing, liquid nitrogen is often used to rapidly freeze tissues after harvest to minimize RNA degradation. However, freezing tissue in liquid nitrogen and subsequent processing is not always convenient, especially if large numbers of samples need to be preserved. Invitrogen™ RNA*later*™ solution is the most cited and trusted tissue storage and stabilization solution that preserves RNA within tissues and cells. The pieces of tissue can simply be dropped into 5 to 10 volumes of RNA*later* solution and stored at 4°C for up to a month prior to RNA isolation.

RNA samples

Small amounts of RNases that may copurify with isolated RNA can compromise downstream applications. Such contamination can also be introduced from tips, tubes, and other reagents used in these procedures. RNase inhibitors are commonly used as a precautionary measure in most enzymatic manipulations of RNA to keep such contaminants in check. Invitrogen™ SUPERase•In™ RNase inhibitor inhibits RNases A, B, C, T1, and 1.

Plasmid preparations

Plasmid DNA used for *in vitro* transcription and coupled transcription-translation reactions can introduce RNase contamination into reactions. Many researchers degrade the RNA in plasmid preps by RNase treatment. If this procedure has been used, we recommend a Proteinase K treatment followed by a phenol-chloroform extraction to eliminate all traces of RNase prior to subsequent reactions. If the DNA template has been linearized by restriction enzyme digestion, a similar treatment is recommended, since restriction enzymes may be contaminated with RNases.

Note: It is highly recommended that a dedicated space be set aside for procedures that require RNase treatment to avoid inadvertent exposure of RNA samples to RNase.

Enzymes

Both commercially purchased and laboratory-prepared enzymes can be a potential source of RNase contamination. At Thermo Fisher Scientific, we have used Invitrogen™ RNaseAlert™ reagents to determine the extent of RNase contamination in numerous commercially available enzymes. It is important that you use only enzymes that are RNase-free when working with RNA. We offer RNase-free enzymes for different kinds of RNA analysis experiments.

RNA storage

The presence of trace amounts of RNase can compromise RNA integrity, even if the samples are stored frozen in an aqueous environment. For short-term storage, RNA samples can be resuspended in RNase-free water (with 0.1 mM EDTA) or TE buffer (10 mM Tris, 1 mM EDTA) and stored at –80°C. Using a buffer solution that contains a chelating agent is a better way to store RNA. Chelation of divalent cations such as Mg^{2+} and Ca^{2+} will prevent heat-induced strand scission (RNA can be chemically cleaved when heated in the presence of Mg^{2+}). We offer nuclease-free water and a variety of Invitrogen™ buffers, including TE, 0.1 mM EDTA, and THE RNA Storage Solution (which has the added benefit of a low pH), for storing RNA. All of these buffers are subjected to rigorous quality control procedures so that they will be nuclease-free.

The best method to preserve isolated RNA for long-term storage is to perform a salt/alcohol precipitation on pre-aliquoted sample and store the nucleic acid as a precipitate in this solution at –20°C. The low temperature and the presence of alcohol inhibit all enzymatic activity. The lower than neutral pH (due to the presence of sodium acetate or ammonium acetate) also helps stabilize the RNA. Note that the RNA will have to be centrifuged out of this solution prior to any downstream application. Another option

is to resuspend the RNA in RNA*secure* Resuspension Solution, which contains an RNase-inactivating reagent. After the addition of RNA*secure* solution, simply heat the sample at 60°C for 10 minutes to inactivate any RNases. If contamination of the sample is suspected at a later date, reheating will inactivate any new contaminants.

Chemical nucleases

Though RNase contamination is most commonly suspected whenever RNA degradation is observed, RNA molecules can also undergo strand scission when heated in the presence of divalent cations, such as Mg^{2+} or Ca^{2+}, at >80°C for 5 minutes or more. Thus, a chelating agent should be present whenever there is a requirement for heating RNA. THE RNA Storage Solution has been expressly designed for storing RNA. It contains 1 mM sodium citrate, which is an efficient chelator of divalent cations, and has a relatively low pH (~6.4) that minimizes RNA base hydrolysis.

Detecting RNase contamination

While contaminating RNase can result in a failed experiment, it is often difficult and time-consuming to determine which solution or piece of equipment is responsible.

Buffers, solutions, and surfaces

Tracing and identifying the source of RNase contamination can be a frustrating and time-consuming endeavor. The easiest but most expensive solution to the problem, especially if a reagent is suspected as the source of the contamination, is to discard all the existing stocks of reagents and start with fresh, RNase-free batches. Another option is to test the suspected solutions for the presence of RNases. Testing can be performed using the Invitrogen™ RNaseAlert™ Lab Test Kit. This kit allows researchers to identify contaminated reagents and equipment quickly, and nonisotopically. In the RNaseAlert Lab Test Kit procedure, an optimized RNA oligonucleotide, double-labeled with both fluorescent and quenching moieties, is introduced as a target for any contaminating RNase. In the presence of RNase, the substrate is cleaved, releasing the fluorescent label. The fluorescence signal can be detected by eye or with a fluorometer. The same technique can also be easily adapted for detecting RNase contamination in tips, tubes, glassware, or any other surface.

RNA samples

RNases can be introduced into RNA samples during RNA isolation (e.g., when small amounts of RNases are carried over into the preparation) or during normal day-to-day use, which inevitably leads to repeated opening and closing of sample tubes and insertion of possibly contaminated pipette tips. RNase contamination is generally detected by looking for degradation of the RNA within the sample.

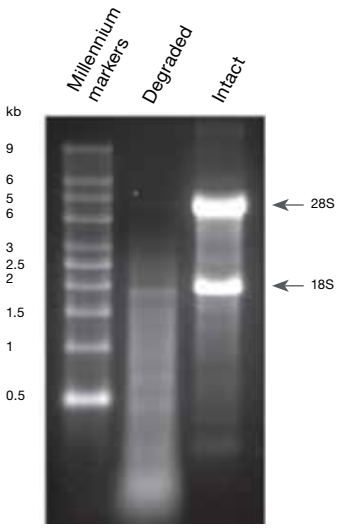

Figure 1. Intact vs. degraded RNA. Degraded and intact total RNA (2 µg each) were run beside Invitrogen™ Millennium™ RNA Markers on a 1.5% denaturing agarose gel. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample. The degraded RNA appears as a lower molecular weight smear.

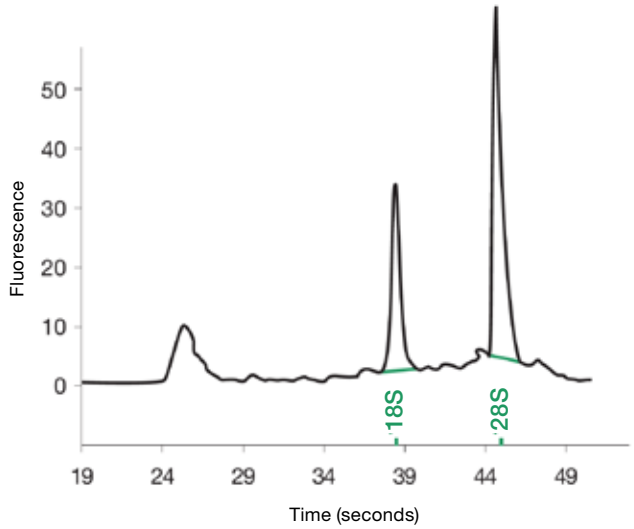

Figure 2. Agilent 2100 Bioanalyzer data. Electropherogram of a high-quality eukaryotic total RNA sample. The 18S and 28S peaks are clearly visible at 39 and 46 seconds, respectively. The microchannels of the 2100 Bioanalyzer instrument are filled with a sieving polymer and fluorescent dye. Samples are detected by their fluorescence, and the signals are translated into electropherograms or into gel-like images (data not shown).

Total RNA samples can be analyzed by running the sample (2 to 5 µg) on a denaturing agarose gel and staining with ethidium bromide (Figure 1), or on the Agilent™ 2100 Bioanalyzer™ instrument (Figure 2). Intact total RNA samples should exhibit a 2:1 ratio of 28S to 18S ribosomal RNA band intensities. A ribosomal ratio significantly lower than 2:1 is usually a sign of degradation. The integrity of RNA can also be analyzed using the Invitrogen™ Qubit™ RNA IQ Assay Kit, which provides a fast, simple method to check whether an RNA sample has degraded, using the Invitrogen™ Qubit™ 4 Fluorometer (required). The assay

utilizes two unique dyes: one that binds to large, intact and/or highly structured RNA (mRNA, tRNA, rRNA), and another that selectively binds to small and/or, degraded RNA. Together they enable you to quickly assess the quality and integrity of an RNA sample in as little as 5 to 10 minutes.

Assessing the integrity of poly(A) RNA samples can be accomplished by performing a northern blot analysis, using a probe against a housekeeping gene such as GAPDH, cyclophilin, or beta-actin. Since the RNA sample is resolved by size on a denaturing gel, degradation manifests as a smear below the full-length transcript.

The severity of degradation will be indicated by an increase in low molecular weight smearing. Absence of a band at the expected full-length transcript size or observation of a smear at the bottom of the gel is an indication that the RNA is very severely degraded.

Degraded RNA samples are unsuitable for northern blot analysis, RACE protocols, or full-length cDNA library construction. However, unless severely degraded, samples may not be affected in reverse transcription quantitative PCR (RT-qPCR) analysis if small amplicons are being analyzed. Samples with some degree of degradation may be appropriate for other types of analysis, such as next-generation sequencing, and for nuclease protection assays (NPAs). RNA degradation in some types of samples (such as formalin-fixed, paraffin-embedded samples or ancient samples) is unavoidable.

Inhibiting RNases in enzymatic reactions

The traditional method for combating RNases in enzymatic reactions such as *in vitro* transcription, reverse transcription, and translation is to use human placental ribonuclease inhibitor (also known as RNase inhibitor protein, RI, or hPRI). This protein is an inhibitor of only the RNase A family of ribonucleases, which includes RNases A, B, and C. The mode of inhibition is noncompetitive, i.e., the protein does not destroy these RNases, but binds them in a 1:1 ratio. A potential problem with the inhibitor is that it might be contaminated with the very RNases that it is designed to inhibit, due to copurification of nucleases. Extended incubation of contaminated preparations of this protein has the potential to slowly release nuclease into enzymatic reactions. Thus, even though this inhibitor can be useful in solving many ribonuclease contamination problems, it is not necessarily the best inhibitor available.

SUPERase•In RNase inhibitor is a broad-spectrum RNase inhibitor that protects RNA against the RNase A family of nucleases as well as against RNase T1 and RNase I. It is recommended as a superior alternative to placental ribonuclease inhibitor (RI or hPRI) in any enzymatic reaction. Invitrogen™ Ambion™ cloned RNase Inhibitor is useful whenever the integrity of RNA must be maintained maintained, such as in *in vitro* reverse transcription, transcription, and protein synthesis. We also offer the RNA*secure* reagent, a nonenzymatic alternative for use in reactions such as *in vitro* transcription, RT-qPCR, and translation.

With all the precautions shared in this note for avoiding, detecting, and inhibiting RNases, you can be more confident when working with RNA.

References

1. Gupta SK, Haigh BJ, Griffin FJ, Wheeler TT. (2013) The mammalian secreted RNases: mechanisms of action in host defence. *Innate Immun.* 19:86-97.
2. Zasloff M. (2009) Antimicrobial RNases of human skin. *J Invest Dermatol.* 129:2091-2093.
3. Klink TA, Woycechowsky KJ, Taylor KM, Raines RT. (2000) Contribution of disulfide bonds to the conformational stability and catalytic activity of ribonuclease A. *Eur J Biochem.* 267:566-572.
4. Miyamoto T, Okano S, Kasai N. (2009) Irreversible thermoinactivation of ribonuclease-A by soft-hydrothermal processing. *Biotechnol Prog.* 25:1678-1685.