

OLSON LAB PROTOCOL: Working with RNA

Revision: 1 / Date: 11.06.2013 / by: PDO / Modified by: PDO

- **RNA is an intermediary molecule that is designed to be quickly degraded, thus working with RNA requires more stringent working practices than DNA**
- **Good working practices with RNA and the reasons for them are explained clearly in the guidance notes provided by Qiagen and reproduced below: reading this guide thoroughly will increase success rates at the bench and give you a good understanding of the causes of problems**
- **ALWAYS wear gloves when working with RNA to avoid endogenous RNases**
- **Other common sources of environmental RNases include those of air-borne bacteria and fungi (see below); thus avoid leaving tubes open on bench any longer than necessary**
- **Use filter tips when working with RNA to avoid introducing air-borne contaminants**
- **Use „DNA LoBind“ Eppendorf tubes throughout; these help reduce RNA sticking to the sides of the tubes, thus increasing yield**
- **Perform all steps and keep all tubes and reagents on ice unless indicated otherwise**

About RNA

RNA is a single stranded molecule comprised of the same four bases as DNA except for uracil which is used instead of thymidine. Up to 85% of the total RNA in a cell consists of non-coding species, such as ribosomal RNA, transfer RNA and micro RNAs. These types of RNAs are not translated into proteins, but are nevertheless able to act as functional enzymes or substrates for gene translation or regulation. As such, they are expressed in effectively all cells at all times (commonly referred to as ‚housekeeping‘ genes).

RNAs for coding genes are called messenger RNAs (mRNA) and consist of all of a gene's exons spliced together into a contiguous sequence of RNA complementary to the DNA sequences. In addition, mRNAs (also called gene ‚transcripts‘) also contain 5' and 3' untranslated regions (UTRs) containing information needed to translate and/or regulate the transcripts (such as microRNAs). Lastly, the 5' end of mRNAs is ‚capped‘ to protect the transcript from degradation and a string of A's is added to the 3' end of the transcript (i.e. the poly-A tail). This latter feature allows for oligo-T primers to selectively target all mRNAs at the exclusion of non-coding RNAs (which lack poly-A tails).

Because mRNAs are simply intermediaries that carry the gene information from transcription in the nucleus to the ribosomes where the genes are translated into proteins, they are designed to be ephemeral and are thus inherently highly unstable in comparison to DNA.

Thinking in terms of gene expression

While working with RNA poses technical challenges beyond those of working with DNA, it also requires **a different way of thinking**. Whereas a genomic DNA (gDNA) sample derived from effectively any tissue type, life stage or developmental state will contain the same genomic information as any other sample from the same organism (i.e. the entire genome, with samples differing only in quantity of gDNA), RNA samples contain copies of only those transcripts that are being expressed at a particular time and in a particular tissue. Obviously this needs to be considered with respect to the genes of interest (GOI), and an appropriate tissue sample chosen for RNA extraction.

We are in an unusually fortunate position of having not only the full genome of our model organism, *Hymenolepis microstoma*, including gene models of all 10,000+ coding genes, but also full transcriptome characterisations from 5 different samples (each replicated 3X): 1) 5-day old larvae (ie. mid-metamorphosis); 2) whole, gravid adult worms; 3) scolex and neck region; 4) mid strobila region (representing proglottid and gamete development), and 5) end region (representing embryogenesis). The transcriptome samples are represented as RNAseq data (ie. fragments of RNA sequence mapped to the genome) and are expressed as FPKM values (i.e. fragments per thousand bases of genome sequence per 1 million mapped reads; a metric that normalises for differences in gene lengths and starting sample concentrations). We also have statistical tests comparing all combinations of samples, providing information on which and where genes are up-regulated during development. Armed with this information, we can easily gauge which genes to study in which stages of development (also helping to deduce the roles of the genes), and have good estimates of the relative levels of expression, and how these levels may bear on our ability to amplify and detect their spatial expression via WMISH.

NOTES from the Qiagen RNeasy Mini Handbook 09/2010

Handling and storing starting material

RNA in animal and plant tissues is not protected after harvesting until the sample is treated with RNAlater RNA Stabilization Reagent (animal tissues only), flash-frozen, or disrupted and homogenized in the presence of RNase-inhibiting or denaturing reagents. Otherwise, unwanted changes in the gene expression profile will occur. It is therefore important that tissue samples are immediately frozen in liquid nitrogen and stored at -70°C , or immediately immersed in RNAlater RNA Stabilization Reagent. The procedures for tissue harvesting and RNA protection should be carried out as quickly as possible. Frozen tissue samples should not be allowed to thaw during handling or weighing. After disruption and homogenization in Buffer RLT (lysis buffer), samples can be stored at -70°C for months. Animal and yeast cells can be pelleted and then stored at -70°C until required for RNA purification.

Disrupting and homogenizing starting material

Efficient disruption and homogenization of the starting material is an absolute requirement for all total RNA purification procedures. Disruption and homogenization are 2 distinct steps:

Disruption: Complete disruption of cell walls and plasma membranes of cells and organelles is absolutely required to release all the RNA contained in the sample. Different samples require different methods to achieve complete disruption. Incomplete disruption results in significantly reduced RNA yields.

Homogenization: Homogenization is necessary to reduce the viscosity of the lysates produced by disruption. Homogenization shears high-molecular-weight genomic DNA and other high-molecular-weight cellular components to create a homogeneous lysate. Incomplete homogenization results in inefficient binding of RNA to the RNeasy spin column membrane and therefore significantly reduced RNA yields. Some disruption methods simultaneously homogenize the sample, while others require an additional homogenization step. Table 3 (page 21) gives an overview of different disruption and homogenization methods, and is followed by a detailed description of each method. This information can be used as a guide to choose the appropriate methods for your starting material.

Note: After storage in RNAlater RNA Stabilization Reagent, tissues become slightly harder than fresh or thawed tissues. Disruption and homogenization of these tissues, however, is usually not a problem.

Appendix A: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice when aliquots are pipetted for downstream applications.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Nondisposable plasticware

Nondisposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 64). Alternatively, chloroform-resistant plasticware can be rinsed with chloroform* to inactivate RNases.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent,* thoroughly rinsed, and oven baked at 240°C for at least 4 hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate). Fill glassware with 0.1% DEPC (0.1% in water), allow to stand overnight (12 hours) at 37°C, and then autoclave or heat to 100°C for 15 minutes to eliminate residual DEPC.

Electrophoresis tanks

Electrophoresis tanks should be cleaned with detergent solution (e.g., 0.5% SDS),* thoroughly rinsed with RNase-free water, and then rinsed with ethanol† and allowed to dry.

Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC

will react with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO₂. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated 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. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

Note: RNeasy buffers are guaranteed RNase-free

Appendix B: Storage, Quantification, and Determination of Quality of RNA

Storage of RNA

Purified RNA may be stored at –20°C or –70°C in RNase-free water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A₂₆₀) in a spectrophotometer (see “Spectrophotometric quantification of RNA” below). For small amounts of RNA, however, it may be difficult to determine amounts photometrically. Small amounts of RNA can be accurately quantified using an Agilent® 2100 bioanalyzer, quantitative RT-PCR, or fluorometric quantification.

Spectrophotometric quantification of RNA

To ensure significance, A₂₆₀ readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml (A₂₆₀=1 → 44 µg/ml). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH.* As discussed below (see “Purity of RNA”, page 66), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity. When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH, 1 mM EDTA,* followed by washing with RNasefree water (see “Solutions”, page 64). Use the buffer in which the RNA is diluted to zero the spectrophotometer. An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 µl

Dilution = 10 µl of RNA sample + 490 µl of 10 mM Tris·Cl,* pH 7.0 (1/50 dilution)

Measure absorbance of diluted sample in a 1 ml cuvette (RNase-free)

A₂₆₀ = 0.2

Concentration of RNA sample = 44 µg/ml x A₂₆₀ x dilution factor
 = 44 µg/ml x 0.2 x 50
 = 440 µg/ml

Total amount = concentration x volume in milliliters
 = 440 µg/ml x 0.1 ml
 = 44 µg of RNA

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A₂₆₀/A₂₈₀) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. However, the A₂₆₀/A₂₈₀ ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A₂₆₀/A₂₈₀ ratio can vary greatly. Lower pH results in a lower A₂₆₀/A₂₈₀ ratio and reduced sensitivity to protein contamination.* For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an A₂₆₀/A₂₈₀ ratio of 1.9–2.1† in 10 mM Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution used for dilution. For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A₂₆₀ reading of 1 = 44 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see “Spectrophotometric quantification of RNA”, above).

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. While RNeasy Kits will remove the vast majority of cellular DNA, trace amounts may still remain, depending on the amount and nature of the sample. For analysis of very low abundance targets, any interference by residual DNA contamination can be detected by performing real-time RT-PCR control experiments in which no reverse transcriptase is added prior to the PCR step. To prevent any interference by DNA in real-time RT-PCR applications, such as with ABI PRISM® and LightCycler instruments, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified. QuantiTect Assays from QIAGEN are designed for real-time RT-PCR analysis of RNA sequences (without detection of genomic DNA) where possible. For real-time RT-PCR assays where amplification of genomic DNA cannot be avoided, we recommend using the QuantiTect Reverse Transcription Kit for reverse transcription. The kit integrates fast cDNA synthesis with rapid removal of genomic DNA contamination.

* Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *BioTechniques* **22**, 474.

† Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.

For other sensitive applications, DNase digestion of the purified RNA with RNase-free DNase is recommended. A protocol for optional on-column DNase digestion using the RNase-Free DNase Set is provided in Appendix D (page 69). The DNase is efficiently washed away in subsequent wash steps. Alternatively, after the RNeasy procedure, the RNA eluate can be treated with DNase. The RNA can then be repurified according to the RNA cleanup protocol (page 56), or after heat inactivation of the DNase, the RNA can be used directly in downstream applications. The protocol for purification of cytoplasmic RNA from animal cells (available at www.qiagen.com/literature/protocols/RNeasyMini.aspx) is particularly advantageous in applications where the absence of DNA contamination is critical, since intact nuclei are removed. Using this protocol, DNase digestion is generally not required: most of the DNA is removed with the nuclei, and RNeasy technology efficiently removes nearly all of the remaining small amounts of DNA without DNase treatment. However, even further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundance target). Using the cytoplasmic RNA protocol with optional DNase digestion results in undetectable levels of DNA, even in sensitive quantitative RT-PCR analyses.

Integrity of RNA

The integrity and size distribution of total RNA purified with RNeasy Kits can be checked by denaturing agarose gel electrophoresis and ethidium bromide* staining or by using an Agilent 2100 bioanalyzer. The respective ribosomal RNAs should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S RNA should be approximately 2:1. If the ribosomal bands or peaks of a specific sample are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the sample suffered major degradation either before or during RNA purification.