

ISOLATION OF RNA AND ITS QUANTIFICATION

Introduction

- ▣ RNA is required for performing various molecular techniques such as reverse transcription real-time PCR (RT-qPCR), transcriptome analysis using next-generation sequencing, array analysis, digital PCR, northern analysis, and cDNA library construction.
- ▣ Obtaining high-quality RNA is the first, and often the most critical, step in performing these techniques
- ▣ Generating RNA, for sensitive and biologically relevant results is most important.
- ▣ So, the RNA isolation procedure must include some important steps before, during, and after the actual RNA purification

Optimizing RNA Preparation and Analysis

- ▣ For optimizing RNA preparation and analysis the steps are:
 - Treatment and handling of samples prior to RNA isolation
 - Choice of technologies used to prepare the RNA
 - Storage of the prepared RNA sample
- ▣ Most traditional RNA purification procedures take place in the presence of RNase inhibitory agents (typically strong denaturants like guanidine salts, sodium dodecylsulfate (SDS), or phenol-based compounds, designed to lower the risk of RNA degradation in a sample).
- ▣ However, it is typically prior to and after the extraction when RNA integrity is at highest risk.

- ▣ RNA can be isolated from cells or tissues like bones, roots, capsules of gram positive bacteria, spores, yeast, etc
- ▣ For maximizing the yield and quality of RNA preparation finding the most appropriate method of cell or tissue disruption for specific starting material is important.
- ▣ During sample disruption for RNA isolation, it is crucial that the lysis agent or denaturant be in contact with the cellular contents at the moment that the cells are disrupted. This workflows prevent processing immediately after collection or when samples are numerous
- ▣ A common solution to these problems is to freeze the tissue/cells in liquid nitrogen or on dry ice. The frozen samples are often preprocessed to select a desired mass or to partially pulverize the sample before exposure to denaturant.

RNA isolation procedure for tissue

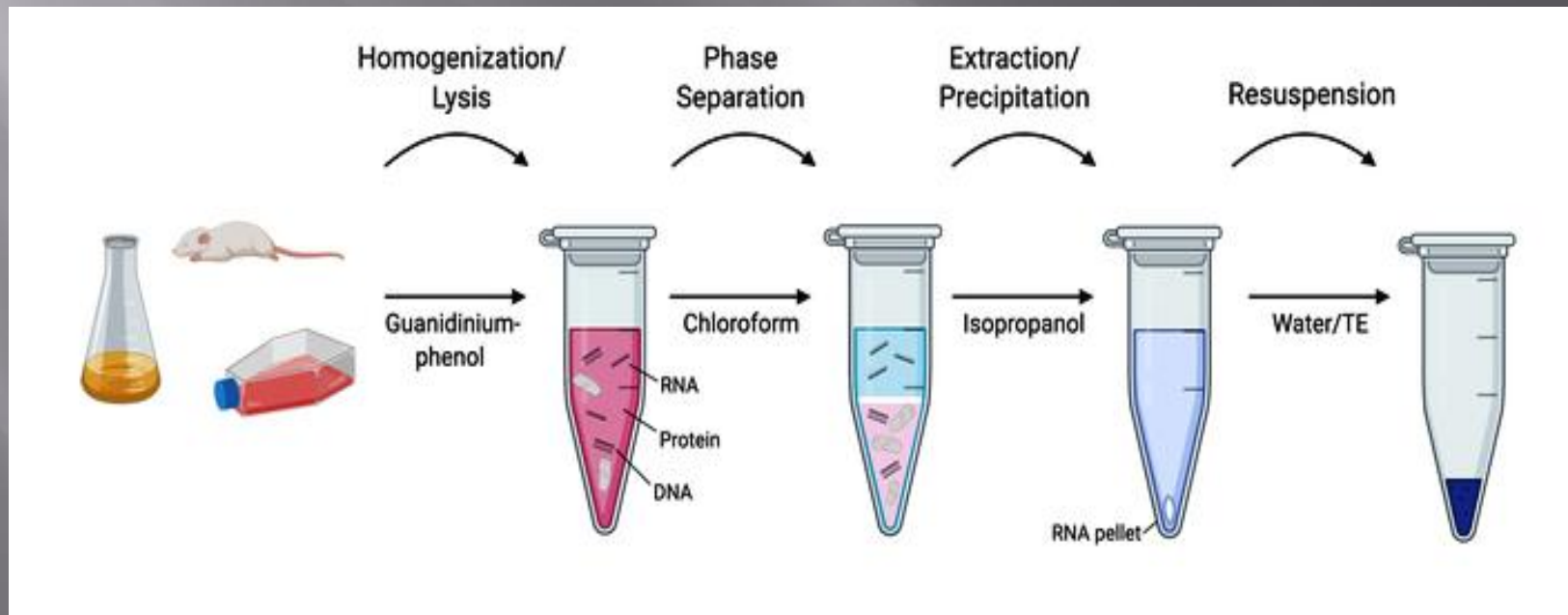
- ▣ Add 1 ml TRIzol to a sterile culture tube (preferably 12x75 mm).
- ▣ To this tube, add the frozen tissue (try not to add more than approximately 20 mg).
- ▣ On ice, pulverize the tissue with a homogenizer at a setting of 25 out of 30 for a total of 2 X 10 sec.
- ▣ Then pour the TRIzol solution into a 1.5 ml Eppendorf tube and proceed as above
- ▣ Leave at room temperature for 5 min.
- ▣ Add 250 μ l chloroform and shake the tube vigorously for about 15 sec.
- ▣ Leave at room temperature for 5 min.

- ▣ Centrifuge at 10,000 rpm for 5 min.
- ▣ At this point, there will be three layers in each tube:
 - Top layer: clear, aqueous
 - Middle layer/interphase: white precipitated DNA
 - Bottom layer: pink organic phase
- ▣ Carefully remove the aqueous phase using a pipette. With a larger pipette, it is harder to control the rate and force of fluid withdrawal and this increases the likelihood of drawing some of the organic or DNA phase.
- ▣ Leave behind some of the aqueous phase (about 1 mm above DNA layer to prevent DNA contamination). Place in another 1.5 ml Eppendorf tube.

- ▣ Add 550 μ l isopropanol to the aqueous phase and mix gently. Leave at room temperature for 5 min.
- ▣ Centrifuge at maximal speed (14,000 rpm) for 20 min. If a low yield is expected, centrifuge for 30 min.
- ▣ Place samples on ice. There should be a pellet barely visible at the base of each tube.
- ▣ Pour off the isopropanol and add 1 ml 75% ethanol in DEPC treated H₂O. Mix gently. Recentrifuge at 9,500 rpm for 5 min.
- ▣ Pour off the ethanol and let the pellets air-dry.

- ▣ This is a critical step; if the pellets dry out too much, the RNA crystallizes and is very difficult to resolubilize.
- ▣ If not enough of the ethanol evaporates, this also prevents the RNA from going into solution.
- ▣ After pouring off the bulk of the ethanol wash, there will be approximately 30–40 μl left in the bottom of the Eppendorf tube.
- ▣ To quicken the evaporation, centrifuge the tubes briefly to force remaining fluid on the side of the tube to the bottom, then pipette off as much of the ethanol as is feasible.
- ▣ The best time to add DEPC treated water to the RNA pellet is when there is only a tiny meniscus of solution left around the pellet itself.

- ▣ Add approximately 15–25 μl (depending on yield) of either DEPC treated TE buffer or water to the RNA pellet.
- ▣ To a small Eppendorf tube, dilute the RNA 1/40 (1.2 μl in 48.8 μl of TE buffer) and add to a microcuvette (path length = 1 cm).



Quantitation of Isolated RNA

- ❑ RNA quantitation is an important and necessary step prior to most RNA analysis methods.
- ❑ UV spectroscopy is the most widely used method to quantitate RNA
- ❑ The traditional method for assessing RNA concentration and purity is UV spectroscopy.
- ❑ The absorbance of a diluted RNA sample is measured at 260 and 280 nm.
- ❑ The nucleic acid concentration is calculated using the Beer-Lambert law, which predicts a linear change in absorbance with concentration

- ▣ Beer-Lambert Law for calculating UV absorbance by nucleic acid.
- ▣ $A = \epsilon CI$ [A=absorbance at a particular wavelength; C = concentration of nucleic acid; I = pathlength of the spectrophotometer cuvette; ϵ = extinction coefficient]
- ▣ Using this equation, an A_{260} reading of 1.0 is equivalent to ~40 $\mu\text{g/mL}$ single-stranded RNA.
- ▣ The A_{260}/A_{280} ratio is used to assess RNA purity. An A_{260}/A_{280} ratio of 1.8–2.1 indicates highly purified RNA.

(A)

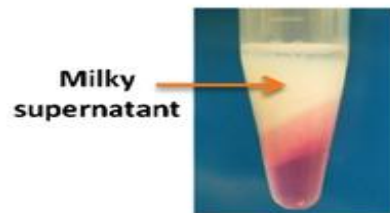
After first phase separation



(B)

After first phase separation

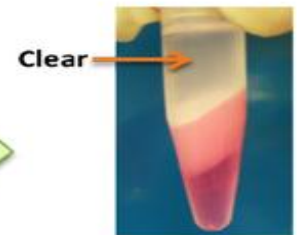
When proteoglycan content is high,



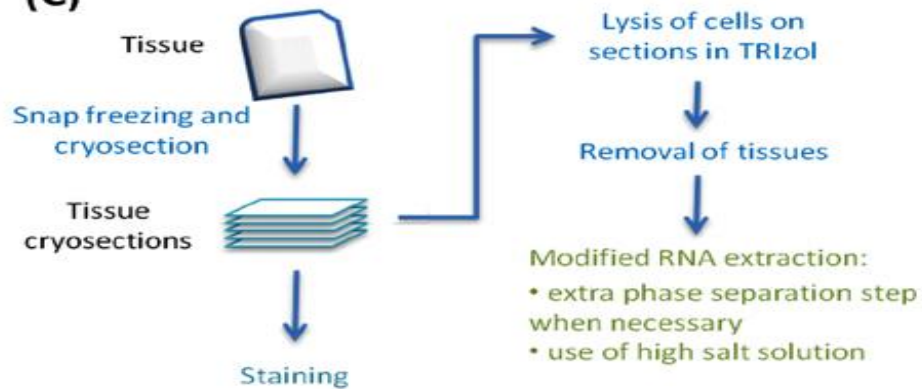
Second phase separation



After second phase separation



(C)



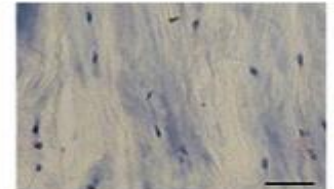
(D)

Staining

Bovine NP



Bovine AF



- ▣ The A_{260}/A_{280} ratio is dependent on both pH and ionic strength.
- ▣ As pH increases, the A_{280} decreases while the A_{260} is unaffected.
- ▣ This results in an increasing A_{260}/A_{280} ratio.
- ▣ Because water often has an acidic pH, it can lower the A_{260}/A_{280} ratio.
- ▣ A buffered solution with a slightly alkaline pH, such as TE (pH 8.0), as a diluent (and as a blank) is recommended to assure accurate and reproducible readings.

- ▣ Then measure the absorbance at 260 nm. The 260/280 ratio should be greater than 1.8.
- ▣ If less than 1.5–1.6 or so, the RNA is likely to be at least partially degraded.
- ▣ Lower ratios also suggest DNA or thiocyanate contamination
- ▣ The concentration is essentially the equivalent of the OD at 260 nm (in $\mu\text{g}/\mu\text{l}$).

Workflow scheme

