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THE MOST COMMON FACTORS THAT REDUCE THE QUALITY OR QUANTITY OF ISOLATED RNA

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The three steps in RNA isolation procedures where errors most commonly cause RNA degradation and loss of recovery are during (1) tissue harvesting and homogenization or freezing, (2) separation of RNA from DNA and protein contamination and (3) RNA solubilization and storage.

CELL LYSIS AND TISSUE HOMOGENIZATION

To recover good quality RNA,, homogenization must be rapid and highly efficient because this step is the most probable area where RNA degradation occurs. Any delay in tissue homogenization and cell lysis allows RNA to remain trapped within cells and provides time for RNase to degrade RNA, reducing both RNA quality and quantity.

Sample Collection

To preserve RNA integrity, the tissue sample must be immediately homogenized in an extraction solution that blocks RNase activity, or immediately frozen for later processing. For best results when freezing tissue, immediately immerse it in liquid nitrogen. Any delay allows RNase-induced degradation and potential modification of gene expression. Liquid nitrogen ensures instantaneous and uniform freezing of the tissue. Freezing on dry ice may lead to temperature gradients in the tissue and reduced RNase inhibition. Tissues with high RNase activity such as pancreas are sensitive to these conditions. Whether immediately homogenizing a tissue sample or freezing it for later use, the time between tissue harvesting and homogenization or freezing should be measured in seconds not minutes.

Tissue Homogenization

When processing fresh tissue samples without freezing, make sure the tissue completely disperses during homogenization within seconds after harvesting. Cell membranes must lyse to allow rapid denaturation of enzymes by the extraction solution. One option is to pre-weigh the approximate amount of reagent required in the homogenization tube. After tissue harvest, immediately place the tissue into the apparatus and homogenize. Weigh the subsequent homogenate to determine the tissue weight and add additional extraction solution to obtain the optimal sample-to-reagent ratio.

Fresh tissues with a high connective tissue content such as heart, artery, muscle, skin and fascia are difficult to homogenize. However, freezing them in liquid nitrogen prior to homogenization makes them easy to fracture and crush, promoting fast and efficient homogenization of these difficult samples.

Tissues rapidly frozen in liquid nitrogen may be stored for long periods at -80 C. Although freezing and storing samples below -50° C will inhibit RNase activity, it is critical the tissue remain frozen until homogenization denatures the RNase enzyme. Avoid any opportunity for thawing. When possible, use liquid nitrogen for tissue manipulation and/or transport between a freezer and tissue processing. As noted above, dry ice may not adequately maintain a temperature low enough to ensure RNase inhibition. Placing tissue frozen in liquid nitrogen directly into the extraction solution allows homogenization before any opportunity for thawing. Such brittle frozen tissue quickly fractures and homogenizes in an appropriate apparatus. For motorized homogenization, tissue should be small enough to enter the rotating stator for instant pulverization.

SEPARATION OF RNA FROM DNA AND PROTEIN CONTAMINATION

The strong chaotropic chemicals in RNA extraction solutions rapidly denature proteins and destroy enzymatic activity. RNA solubilized in the tissue homogenate is not subject to degradation and can be stored for long periods at -80 C.

RNA isolation methods remove DNA and protein contamination from the water supernatant containing the RNA product. The recommended ratio of tissue - to - reagent volume minimizes the opportunity for DNA or protein to contaminate the isolated RNA. Exceeding this ratio with an excess of tissue leads to DNA and/or protein contamination of the RNA product. Increasing the reagent volume addresses this problem, improving the A260/280 and A260/230 absorbance readings. Spectrophotometric readings do not provide information about DNA contamination of an RNA product. Use either electrophoretic separation or negative-RT PCR of the RNA sample to address this question.

After performing phase-separation (TRI Reagent®) or water precipitation (RNAzol®-RT) steps in RNA isolation protocols, the water supernatant is transferred to a clean tube for RNA precipitation by the addition of alcohol. The water supernatant with RNA contains salt that can become a problem when isolating a very small amount of RNA. In this case, residual contaminants may be relatively high as compared to such a small amount of RNA. After precipitating the RNA into a small, distinct pellet, remove the water - ethanol supernatant from the tube. Centrifuge the RNA pellet for 15 - 30 seconds to collect any

residual supernatant at the bottom of the tube for easy removal by pipetting prior to ethanol washes. This prevents residual contaminants from precipitating with the RNA pellet during ethanol wash steps and improves the final A260/280 and A260/230 spectrophotometric readings for pellets containing < 5 µg of RNA.

RNA SOLUBILIZATION AND STORAGE

Minimally dry RNA pellets prior to solubilization. Pellets usually remain opaque white in appearance; clear pellets often indicate over-drying has occurred. Over-drying causes (a) difficulty solubilzing the RNA product, (b) unsolubilzed RNA resulting in "low yield" readings with decreased A260/280 spectrophotometric reading, and (c) selective and increased solubilization of small RNA species as opposed to large RNA, causing the RNA product to appear degraded.

RNA solubilized in RNase-free water should remain stable for several months when stored at -80 C. Repeated freeze - thaw cycles increase the opportunity for RNase contamination and degradation. Instead of exposing RNA to repeated freeze - thaw cycles, freeze multiple aliquots to avoid repeated handling of the entire sample volume.

When solubilizing small quantities of RNA in small volumes of water (for example < 5 µg RNA in 5 - 10 µl), residual ethanol wash solution can inhibit RNA solubilization. Partially solubilized RNA has lower A260, A260/280 and A260/230 absorbances, suggesting low nucleic acid recovery and potential contamination. The RNA profile appears degraded on a Bioanalyzer because only small RNA is solubilized in the presence of the residual alcohol. To avoid this issue, after removing the second ethanol wash, centrifuge the RNA pellet for 15 - 30 seconds as outlined above. Remove any residual alcohol and dry the RNA pellet for a few minutes prior to solubilization. Allow 5 - 7 minutes for the RNA pellet to dissolve in water at room temperature and then mix the RNA solution with a platform shaker for 5 - 10 minutes to ensure complete solubilization of the RNA.

Alternatively, solubilize RNA in a stabilized formamide solution such as FORMAzol®. RNase is inactive in formamide allowing, allowing short storage at room temperature (hours) and indefinite storage at -20 or -70 C. Precipitate the RNA to remove it from the formamide solution and solubilize in water for use in downstream applications. RNA in formamide may be used directly in reactions for electrophoretic separation of RNA in denaturing agarose gels. When performing spectrophotometric readings, use a solution containing formamide to create an appropriate blank for measurements. Absorbance readings for RNA in formamide are about 10% higher than readings for RNA in water.

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