St. Michael's

Inspired Care.
Inspiring Science.

Quantitative Real Time PCR workflow

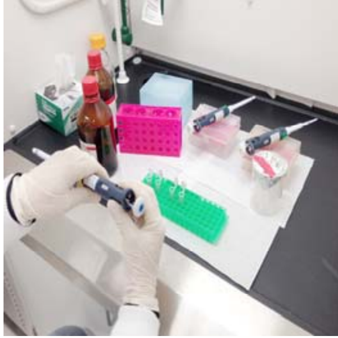
1. RNA isolation

Tissues or cells that have been collected for RNA isolation should be flash frozen in liquid nitrogen and stored at -80 or in RNA storage solution (Ambion's RNAlater, Amersham/Pharmacia RNAguard) for stability. This is especially true for samples that are 1) collected over a long period of time and therefore, cannot be processed at the same time 2) human samples collected through biopsy when processing cannot occur instantaneously 3) ample in number and not collected simultaneously. A mortar and pestle are typically used to crush the sample in liquid nitrogen when the starting material is frozen. RNA can be isolated using a number of different methods but tissue disruption methods are usually mechanical or enzymatic. Which method you choose depends primarily on your sample type but also on your experimental system and downstream applications but regardless, the method has to be very fast and thorough. Slow disruption, for example by placing tissue in guanidinium isothiocyanate (GITC) lysis solution without mechanical disruption may cause the RNA to degrade by endogenous ribonucleases (RNases) that are released and not accessible to the denaturing effects of the GITC. This is especially true for tissues that are enriched in RNases such as the spleen and pancreas.

RNA is very difficult to work with because of the presence of ribonucleases (RNases) EVERYWHERE! RNases are very stable and hard to inactivate, so it is necessary to maintain (as much as possible) an RNase free environment when working with RNA. Here are some tips to consider:

Tips for working with RNA

- Ideally, you will have a well delineated RNA working area in your lab space and you will keep this area as clean as possible and have dedicated RNA ONLY solutions (including DNase/RNase free water), pipettes etc. Ensure that all working surfaces in your RNA zone, including pipettes and anything that you will be in contact with, are wiped down thoroughly with an RNase inactivating solution, such as Rnase ZAP (Ambion) or the homemade version which is 0.5% SDS followed by 3%H_2O_2



- Always wear gloves when you enter into the RNA area (with a lab coat and hair net if you really want to be safe!) and at all times during your RNA isolation to prevent you from contaminating with the RNases that are covering your skin. It's a good idea to double glove and remove the outer gloves when you leave the RNA area and put a fresh pair on when you re-enter

- Splurge and purchase filter/barrier tips. Barrier tips prevent potentially contaminating aerosols from your pipette from reaching your sample/reagents. They are a must-have for RNA work

- Any ceramics (mortar and pestle) and/or glassware that you will use in your isolation procedure need to be baked in an oven at 300°C for 2 hours, or 180 °C overnight to kill any RNases on the surface

- Use RNase-free enzymes. Enzymes isolated from bacteria (e.g. DNase) can be full of RNases. Make sure you use certified RNase-free enzymes on your RNA samples

- Use an RNase inhibitor when it's not possible to keep things completely RNase-free. Protector RNase inhibitor (from Roche) is a good example. Avoid high temperatures (above 60°C) or denaturing conditions that could deactivate the inhibitor

- Store RNA at -80°C. Make aliquots if the sample is to be used a number of times to avoid freeze/thaw cycles

Methods of Disruption for mammalian cells and tissue

- It is recommended to process your samples in small batches of 10-15 for RNA isolation to minimize the exposure and handling time

- Ideally, tissues should be processed fresh (unfrozen) but it is important to keep the tissue on ice and to process it within 30 min after isolation, if it's not put in an RNA guarding solution. When disrupting fresh tissue, the sample should be mechanically sheared immediately when placed in lysis solution (Trizol or other lysis solution) and not left in solution without disruption (see note above on active RNases)

- If it is not possible to isolate RNA immediately from fresh tissue, the sample must be flash frozen and stored at -80 until processing can occur. Frozen tissue is most easily disrupted by grinding in a mortar and pestle (that have been baked to eliminate RNases and cooled to -20 and then to -80).

Tissues should be ground into a fine powder and transferred to Trizol or an appropriate lysis buffer and then homogenized

- Cultured cells are easily disrupted by vortexing, vigorous pipetting, by passage through a needle or lysed directly on the culture plate in Trizol or lysis solution. Ensure that the culture plate is kept on ice during the lysis procedure to ensure that endogenous RNases are inactive
- Following RNA isolation, DNase treatment is highly recommended to eliminate contaminating (and potentially interfering) genomic DNA. Many protocols (especially kits) involve a DNase treatment step, so be sure to review your protocol and include this important step either during the isolation procedure or following RNA isolation