Cell sorting for RNA isolation

Adapted from cyto.purdue.edu and University of Toronto

Please consult with the microarray and flow cytometry staff for successful RNA recovery from cells sorted using flow cytometry. Several key points are highlighted below:

Instrument decontamination:

Preparing the flow cytometer is often not a small task as it should be completely free of RNases from the sheath tank to the sorting nozzle before sorting. This decontamination procedure may take considerable time depending on what the flow cytometer has been used for, the age of the instrument, and consequently the contamination level.

Ensure the dip tube, septa, flow cell, all tubing lines, and nozzles have been decontaminated with bleach, RNase ZAP, ethanol, autoclaving, or other qualifying technique prior to the sort. The sheath fluid and tank must be RNase-free as well. An in-line 0.2uM filter is not sufficient for "filtering out" RNases. Immediately before the sorting run, run a tube of 10 % bleach for 300 seconds followed by a minimum of 3 tubes of DEPC water for 180 seconds each (*you are required to provide DEPC water for your sorts as this is a specialized application*).

During the sort, it is recommended to sort directly into your extraction reagent, such as Trizol LS or Qiagen's RLT (when volumes permit…less then or equal to 500ul). When sorting volumes are high [>0.5ml], it maybe necessary to sort into cold media or PBS and spin your cells to a pellet and freeze or extract using standard procedures. If secondary processing is required, it is important to sort into RNase-free tubes and buffers and keep on ice during the sort. Always extract RNA as soon as possible.

As a rule, retain some cells and extract the RNA to determine the condition of RNA prior to the sort.

Trizol ™ LS

When sorting into Trizol, consider using Trizol LS (cat # 10296-010) which is more concentrated than regular Trizol. This formula allows lower quantities of reagent to be used relative to the amount to the sorted sample volume (regular Trizol can tolerate 10% sorted sample or 100ul of total sorted volume to 900 ul of Trizol while TriZol LS allows 30% sample volume). It is rarely applicable to use standard Trizol for most sorting purposes.

Simple Method:

1] Start with 500ul of Trizol LS in a sterile FACS tube.

2] Sort into the tube, periodically mixing if necessary to get liquid off sides of tube and keep evenly mixed.

3] After the sort, using a pipet with sterile tip, measure the final volume.

4] Calculate the exact volume of sample sorted into the Trizol LS.

5] Adjust the amount of Trizol LS required to maintain your sample at less than or equal to 30% of the total volume.

6] Proceed to step 2 of the Trizol protocol: http://www.invitrogen.com/content/sfs/manuals/15596026.pdf

7] For the final precipitation step, consider using the Axygen MCT175 Ultra-clear microfuge tubes for best visualization. A co-precipitate maybe added during the final precipitation such as glycogen or Pellet Paint.

It is not recommended to freeze the sample-Trizol mix. It should be extracted ASAP. Only when absolutely necessary, freezing at -80 is tolerable. We have noted as much as a 50% reduction in RNA recovery when extracting previously frozen cells in extraction buffer.

Qiagen's RNeasy™

In the case of Guanidium Isothiocyanate (or Qiagen's RLT buffer), sorted at a ratio of 100ul of sort sample to 350 ul of RLT or a multiple of that. It should be noted that when using RLT buffer, that recoveries are often half that of Trizol LS and you lose the transcripts that are smaller than 200 bp, but the RNA is much cleaner. This is especially important with miRNA as they are less than 30 bp and fall into to this <200bp category.

Simple Method using the RNeasy Clean-up protocol

1] Start with 500ul of RLT Buffer in a sterile FACS tube.

2] Sort into the tube, periodically mixing if necessary to get liquid off sides of tube and keep evenly mixed.

3] After the sort, using a pipette with sterile tip, measure the final volume.

4] Calculate the exact volume of sample sorted into the RLT.

5] Adjust the amount of RLT required so that there is exactly 350 ul of RLT to every 100 ul of sorted sample volume. (See RNA clean-up protocol in Qiagen handbook)

6] Add 250 ul of 100% ETOH for every 350 ul RLT-100ul sample volume.

7] Mix and apply to a RNeasy mini or RNeasy minelute column in 700ul aliquots and spin at > 8000 g. Additional volumes maybe added to the same columns.

8] If a large amount of preparation is to be added to a column (>5ml), multiple columns maybe used. In this case, the elution of RNA from the columns should be done with the same aliquot of water to maintain a concentrated sample in the final extract. In other word, the 30-50ul of water used to elute your first column will be used to elute your second column and third column. This will maintain your RNA in one 30-50 ul aliquot. This is only necessary for sample containing low concentrations of RNA.

9] DNase treatment is optional, but expect a 20-30% loss in RNA recovery.

Notes on RNeasy technique:

Note 1: If the sorted volume is less than 100 ul add DEPC H20 so the amount of sorted sample volume and DEPC is 143 ul. This maintains the 3.5 RLT to 1 aqueous ratio (500/143=3.5). The volume of ETOH must also be adjusted.

Note 2: The RNeasy Mini kit (74103) is designed to elute the RNA in 30-50ul of final volume and is better suited for higher RNA amounts (i.e. >20,000 cells). For fewer cells, the RNeasy MinElute (74204) is better suited as the final RNA is eluted in 14ul. Both allow an optional DNase treatment step.

Note 3: It is not recommended to freeze the sample sample-RLT mix. It should be extracted ASAP. Only when absolutely necessary, freezing at -80 is tolerable. We have noted a 80% reduction in RNA recovery when extracting previously frozen cells in RLT extraction buffer.

Other Comments:

1] Using control cells [non-critical cells], conduct a test run through the entire procedure. Do not start with your "good" preps. Quantitate the RNA with the Nanodrop ND1000 and evaluate using the Agilent 2100 bioanalyzer. If quantitation is too low for a Nanodrop reading, run on the bioanalyzer Picochip.

2] Freezing cells in Trizol at −80 is not recommended but is tolerable. Expect a 50% reduction in RNA recovery. This is not the case with Qiagens RLT buffer. We do not recommend freezing RLT with your cells.

3] In rare cases, the flow cytometer, even after a good decontamination procedure can still be a source of RNases. If possible you may wish to consider suspending cells in RNAlater prior to the sort. If RNAlater is not an option, you may consider adding an RNase inhibitor to cell suspension. Of course this is volume dependent and may not be economically realistic.

4] When not sorting directly into extraction reagent, sort into vessels containing media or PBS on ice when applicable. Immediately after the sort, extract RNA according to the selected reagents manufacturer's protocol and evaluate the integrity of the "before" and "after" RNA

5] Keep nozzle pressure low if possible.

6] When staining cells with any stain including mAb, make sure they are RNase-free and the procedures are RNase-free, this includes all rinse buffers and tubes. If necessary, mix some good RNA with your stain and incubate for 5 minutes and run a RNA assessment.

7] Prior to sorting or during a sort, it may be a good idea to stain the cells with a viability dye so dead cells can be rejected. Dead cells can be a large contributor of RNases.

8] Remember DEPC water DOES NOT inactivate RNases, and is only RNase-free water. Use only sterile RNase-free tubes on the cytometer that have never been open to contaminated air.

Article of Interest: Biotechniques. 2002 Apr; 32(4):888-90, 892, 894, 896. High-quality RNA and DNA from flow cytometrically sorted human epithelial cells and tissues. Barrett MT, Glogovac J, Prevo LJ, Reid BJ, Porter P, Rabinovitch PS. Fred Hutchinson Cancer Research Center, Seattle, Washington, US

Reference:

www.cyto.purdue.edu/MD-parts/0cdcc658a615435c17c0270a69a3dc9b793d2461.doc