Chapter 2

Isolation of Total RNA from Transgenic Mouse Melanoma Subsets Using Fluorescence-Activated Cell Sorting

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Abstract

The majority of tumors, including melanoma, are phenotypically heterogeneous in that they contain various cell populations with differential expression of cell surface antigens such as CD133/Prominin-1. We have used fluorescence-activated cell sorting (FACS) technology to purify $CD133^+$ and $CD133^-$ cellular subsets from mouse melanoma models for high-quality total RNA practical for downstream applications such as expression profiling. Implementation of this strategy can lead to higher resolution of transcripts that are potentially important for the survival and functionality of one cancer cell population relative to another. Suboptimal extraction of RNA after FACS is common and can ultimately result in misinterpretations that impede the effective design of novel therapies. Here, we describe a number of methods that have been amenable to the successful isolation of high-quality total RNA after FACS of $CD133^+$ and $CD133^-$ mouse melanoma cell fractions.

Key words: Melanoma, Mouse models of cancer, FACS, Cell surface markers, Cell subsets, RNA isolation, RNA FACS sorting

1. Introduction

Methods for genome-wide expression analyses, such as DNA microarrays (1), can be used to delineate global RNA expression differences between cancer cell subsets that show variations in function such as their abilities to resist chemotherapy or propagate tumors. The cell surface antigen CD133 has been demonstrated to identify cancer cells from a variety of solid-tissue cancers such as melanoma that display higher tumorigenicity or treatment resistance (2–5) and can be characterized through, for example, gene expression profiling of the $CD133^+$ and $CD133^-$ subset phenotypes. To accomplish such a task requires well-established flow cytometric sorting methods and RNA extraction protocols (6, 7).

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It is well known that RNA is a sensitive nucleic acid that can easily degrade as a result of erroneous introduction of ribonucleases (RNases) either from instrumentation, immunostaining procedures, end user, or endogenously from the sample itself (8, 9). In addition, improper extraction and storage of RNA can decrease its overall half-life, compromising future utility. Here we establish several workflows for fluorescence-activated cell sorting (FACS) of $CD133^+$ and $CD133^-$ melanoma cell subsets for high-quality total RNA purification including instrument decontamination, cell surface marker labeling, cell sorting procedures, and RNA handling and extraction methods. In addition, we discuss quantification techniques and integrity analyses used for validating the RNA quality of these cellular subsets after FACS.

2. Materials

2.1. Cell Culture and Antibody Staining

1. Dulbecco's Modified Eagle's Medium-F12, 1:1 (Gibco).
2. Fetal-bovine serum (FBS), US-origin, irradiated, heat-inactivated (Hyclone).
3. Modified Eagle's Medium Nonessential amino acids (Cellgro).
4. Trypsin 0.25%/2.2 mM EDTA (Cellgro).
5. Penicillin streptomycin (Pen/Strep), 1×10^4 U/ml each (Cellgro).
6. Phosphate-buffered saline (PBS), RNase-free (Ambion).
7. Bovine serum albumin (BSA), RNase-free (Equitech-Bio).
8. RNase inhibitor, e.g., RiboLock (Fermentas Corp).
9. Dulbecco's PBS containing 100 U/ml RiboLock (PBS-RIBO).
10. RNase-free 1.5 ml microcentrifuge tubes (Axygen, #MCT175c).
11. RNaseZap (Ambion).
12. Rat anti-CD133 mouse monoclonal antibody (eBioscience).
13. AlexaFluor488 chicken antirat IgG secondary antibody (Invitrogen).

2.2. Flow Cytometry and Sorting

1. BD FACSAria flow cytometer or equivalent.
2. RNase-free water (VWR Scientific).
3. Sterile polystyrene round-bottom tubes for flow 5 ml (BD Falcon).
4. Bleach 10% (0.525% sodium hypochlorite).

5. Sterile sheath fluid (saline) RNase-free.
6. Propidium iodide (1 mg/ml) solution-ultra-high purity (Enzo, #enz-52403R).
7. Bovine serum albumin, RNase-free (Equitech-Bio).
8. RNaseAlert RNase detection system (Ambion).

2.3. RNA Isolation

1. Trizol or Trizol LS or equivalent.
2. RNeasy Micro Kit (Qiagen).
3. Beta-mercaptoethanol.
4. Chloroform (100% ACS Grade).
5. 100% Ethanol (Electron Microscopy Sciences).
6. MaxyClear RNase-free tubes 1.5, 15, and 50 ml (Axygen).
7. QIAvac-24 Plus Vacuum manifold (Qiagen).
8. Nanodrop ND1000 spectrophotometer.
9. Qubit Spectrofluorometer (Invitrogen).
10. Quant-IT RNA reagents (Invitrogen).
11. Agilent 2100 Bioanalyzer or equivalent.

3. Methods

3.1. Quality Control of the Fluorescence-Activated Cell Sorter

Before proceeding with FACS of cell subsets, stringent quality control of the instrumentation is mandatory to ensure the success of good quality RNA isolation from sorted cell populations. This involves thorough decontamination followed by empirical validation of FACS machine sanitation. Decontamination time will depend on the instrument type, age, and degree of contamination. However, procedures for sanitizing any FACS instrument are similar, and so a review of the following steps is warranted. It is urged to perform all steps with RNaseZap-treated gloves in a low contamination environment (see Note 1). Once decontamination is complete, a test sort using noncritical cells with a known viability >80% should be performed to test the instrument (see Note 2).

1. Ensure the dip tube, septa, flow cell, tubing lines, and nozzles have been decontaminated with 10% bleach, 100% ethanol, RNaseZap, autoclaving, or other suitable qualifying technique prior to the sort.
2. Ensure sheath tank and fluid are RNase-free. Quality control sampling of each may be tested with RNase-detecting reagents such as RNaseAlert.

3. Replace all contaminated fluid lines and filters as outlined by the manufacturer.
4. Prior to sort, run several tubes of 10% bleach through the flow cytometer including the sorting components followed by flushing with sterile RNase-free sheath fluid or PBS-RIBO.
5. Perform a sort using a bead solution containing 400 U/ml of an RNase inhibitor just prior to sorting critical samples.

3.2. Preparation of Melanoma Cells and Antibody Staining for FACS

Melanoma cell lines were derived from transgenic conditional mouse melanoma tumors as previously described (10). Tumors were finely minced using aseptic technique and enzymatically dissociated with 0.05% trypsin/0.55mM EDTA for 30 min at room temperature, with thorough mincing every 10 min. Dissociated tumors were then lightly triturated 15–20 times, and the resulting suspensions were transferred to tissue culture treated 10 cm adherent dishes. Melanoma cultures were grown in 1:1 DMEM:F12 media with 5% FCS and 1% Pen/Strep (media complete) in a cell culture incubator at 37°C with 5% CO_2 and allowed to grow until approximately 75% confluent. The following protocol was then followed with proper RNA handling in a biosafety cabinet or PCR hood for indirect antibody labeling of cells for the surface marker CD133 followed by FACS of $CD133^+$ and $CD133^-$ cellular subsets using a BD FACSAria flow cytometer (see Note 3).

1. Aspirate media from 10 cm adherent melanoma culture dishes and detach cells by briefly incubating (2–3 min) with 1 ml of 0.25% trypsin/2.2 mM EDTA, followed by neutralization of trypsin with 10 ml of media complete.
2. Centrifuge cell suspensions at 800×*g* for 5 min, aspirate supernatant, and resuspend cell pellet in 1 ml of 1× PBS-RIBO with 2% BSA (PBS-RIBO-BSA) (see Note 4).
3. Perform a viability count using a hemocytometer and Trypan Blue dead-cell discrimination dye (see Note 5).
4. For each sample and control, transfer 5×10^5 cells to a new tube. Controls should include samples with primary antibody only, secondary antibody only (or isotype-control only), unstained cells, and propidium iodide only. These are required for fluorescent compensation and proper gate positioning.
5. All samples and controls are centrifuged at 800×*g*, aspirated, and resuspended in 100 µl of PBS-RIBO-BSA followed by staining with 1 µg/ml final concentration anti-CD133 primary antibody for 30 min at 4°C (in fridge, not on ice).
6. Samples are quenched with 900 µl PBS-RIBO-BSA, centrifuged, aspirated, and resuspended in 100 µl PBS-RIBO-BSA.

7. Each sample is stained with a species-matched, AlexaFluor-488 chicken antirat IgG secondary antibody at 1:1,000 for 20 min in a dark fridge at 4°C.
8. Centrifuge cells, aspirate supernatant, and resuspend cells in 500 µl PBS-RIBO-BSA with a final concentration of 1 µg/ml RNase-free propidium iodide for dead-cell discrimination. Transfer samples to FACS machine-compatible, sterile 5 ml round-bottom tubes and cap.
9. During the flow cytometric procedure, exclude all propidium iodide-positive signals (i.e., dead cells). Whenever possible, use forward scatter (FSC), side scatter (SSC) height, width, and area measurements to exclude any potential doublets or putative apoptotic/dead cells. Live, single cells are then analyzed and sorted by FACS on CD133 signal into precooled, RNase-free 1.5 ml microcentrifuge tubes for subsequent total RNA extraction of purified cell subsets (see Note 6).

3.3. Methods for Sorting Cell Subsets for Total RNA Extraction

There are a variety of procedures for recovering total RNA from sorted cells. The choice of any one protocol depends on two factors: (1) whether the type of FACS machine used for cell purification is mechanical or electrostatic and (2) whether high or low dispensed sort volumes are expected. Mechanical sorters, such as the BD FACSCalibur, use a mechanical sorting device called the "catcher tube" positioned near the flow cell and sort relatively slowly (e.g., 300 events/s) with a relatively high sort volume (e.g., 100 nl–10 µl per event). Therefore, direct sorting of cells using mechanical sorters is not ideal for sorting large numbers of cells directly into RNA extraction buffer as the high dispensed volumes will dilute the buffer substantially and impede RNA recovery. When using mechanical sorters, it is recommended to first centrifuge the sorted cells to form a cell pellet followed by addition of the chosen RNA extraction buffer as outlined below in Subheading 3.3.1. It is important to consider that any additional handling before adding the RNA extraction buffer, such as centrifuging, may lead to consequential gene expression changes (see Note 7).

Electrostatic sorters or "stream-in-air" FACS machines can operate at much higher speeds (e.g., 25,000 events/s or more) and involve a vibrating nozzle by which cells exit within single droplets resulting in much smaller dispensed sort volumes (11). Electrostatic sorters are also capable of fitting various sized nozzles in order to accommodate for cell size and maximize cell viability during the procedure. For example, a 70 µm nozzle decreases flow stream width, thereby resulting in droplet volumes of 1 nl drops per event – an approach applicable to sorting small cell types (e.g., T lymphocytes). In contrast, a 100 µm nozzle will relax flow stream width slightly to accommodate larger sized cells

(e.g., tumor cells) which results in volumes of 5–10 nl drops per event. Although the exact droplet size may vary slightly based on the system settings of each individual flow cytometer, smaller dispensed volumes allow for sorting directly into RNA extraction buffer such as Trizol LS or RLT buffer from the RNeasy system (see Note 8). Although other alternative methods for RNA isolation from sorted cells are available, they will not be described here (see Note 9).

3.3.1. RNeasy System for RNA Isolation After Centrifugation

Isolation of cells from high sorted volumes, such as those from a mechanical sorter, will require a centrifugation step to collect the cell pellet followed by RNA isolation using a silica column approach, such as the RNeasy microcolumn, or a standard Trizol precipitation method as described by the manufacturer (see Note 10) (12). When sorting cells for RNA, it is important to consider adding an RNase inhibitor to the sort recovery tube prior to the sort and adjust to 5–20 U/ml following the sort whenever possible (see Note 11). Sorting directly into a cell preservation reagent for future RNA isolation should be avoided (see Note 12).

1. Immediately following the sort, aseptically centrifuge cells to a pellet at 1,000 × *g* for 10 min using a refrigerated centrifuge.
2. Using a sterile aspirator, remove all supernatant from the cell pellet.
3. Add 100 µl of RNase-free water and 350 µl of RLT buffer and vortex for 30 s (see manufacturer's protocol) (13).
4. Add 250 µl of 100% EMS grade ethanol and vortex.
5. Using a micropipettor with aerosol resistant tip, transfer sample to the RNeasy microcolumn and centrifuge at >10,000 × *g* for 15 s. Replace waste capture tube containing the pass-through liquid.
6. A DNase treatment (steps 8–10) may be required when downstream methods involving random hexamer priming such as in the case of exon microarrays, RT-qPCR, or equivalent are used. If no DNase treatment is required, proceed to step 10 (see Note 13).
7. Apply 200 µl of RW1 buffer to the column and centrifuge at >10,000 × *g* for 15 s.
8. For each sample, prepare the DNase solution from the Qiagen RNase-free DNase kit by combining 70 µl of RDD buffer with 10 µl of DNase I (27.3 Kunitz units total) and applying 80 µl to the column's silica membrane. Incubate at room temperature for 20 min.
9. Add 200 µl of RW1 buffer to the column and centrifuge at >10,000 × *g* for 15 s. Replace the waste capture tube containing the pass-through liquid.

10. Apply 0.5 ml RPE buffer to the column and centrifuge at >10,000 × *g* for 15 s. Replace the waste capture tube containing the pass-through liquid.
11. Repeat step 10.
12. Using a 20 µl pipette, remove the remaining liquid that may be caught up on the edge of the column's inner O-ring.
13. Perform an extended centrifugation for 3–5 min to remove as much liquid from the membrane as possible. Do not centrifuge with column open as described in the manufacturer's protocol.
14. Replace waste tube with a new standard RNase-free 1.5 ml microcentrifuge tube.
15. Apply 15 µl of 60°C RNase-free water directly to the center of the RNeasy microcolumn membrane and incubate at room temperature for 30 s.
16. Centrifuge at >10,000 × *g* for 15 s.
17. Carefully remove the 15 µl of sample from the tube and reapply it to the same RNeasy membrane again. Close column and centrifuge at >10,000 × *g* for 15 s. This reelution is performed with the same 15 µl aliquot to assure complete recovery of RNA from the entire surface of the column's silica membrane.
18. Remove the RNeasy microcolumn from the microcentrifuge tube containing the 15 µl of sample, and add the equivalent of 20 U of RNase inhibitor and vortex. Store sample at –20°C.
19. Quantify the RNA using a high resolution spectrometer such as the Nanodrop ND-1000 and Qubit fluorometer (see Subheading 3.4.1).
20. Analyze the RNA quality using an Agilent 2100 Bioanalyzer or equivalent (see Subheading 3.4.2).

3.3.2. Direct Sort Method

When low sort volumes are expected, it is advantageous to sort directly into extraction reagent such as Trizol LS or RLT buffer in order to minimize downstream handling and inadvertent gene expression changes. Regardless of the method selected, it is imperative to maintain the exact ratio of aqueous sorted volume to extraction reagent consistent with the manufacturer's recommendations and to extract RNA promptly. If immediate extraction is not possible, then short-term storage in dilute extraction reagent may be considered (see Note 14). Although direct sorting into extraction buffer is optimal for RNA recovery, secondary analyses such as microscopy or postsort cell purity validation will require additional steps (see Note 15).

3.3.2.1. Direct RNA Extraction Using a Combined Trizol LS-RNeasy Method

1. Start with 500 µl of Trizol LS in a sterile RNase-free FACS tube if choosing to sort directly into the Trizol LS. Otherwise, sort into 1× PBS-RIBO and spin cells down at 800 × *g* for 10 min, aspirate supernatant, and then add 500 µl of Trizol LS.
2. Multiple sort tubes may be used to collect cells if dispensed sort volumes exceed the volume capacity of the sort collection tubes. If so, use 500 µl starting Trizol LS volume for the extra sort tubes as well.
3. After the sort, use a pipette equipped with an aerosol resistant tip to measure the final volume in the tube. Subtract the amount of Trizol LS to determine the amount of dispensed liquid.
4. Adjust the amount of Trizol LS required to maintain the sample at a Trizol:dispensed volume ratio of at least 3:1. This may require the solution to be transferred to a larger RNase-free tube (see Note 16 for a mathematical example).
5. Add 200 µl of chloroform for every 750 µl of Trizol LS to the tube and mix. Let the samples sit on bench top for 3 min. Alternative organic phases may be used in place of chloroform but are not preferred by the authors (see Note 17).
6. Centrifuge at >10,000 × *g* for 10 min at 4°C to separate the top aqueous layer from the bottom layer and interface. If the volume of solution is too large to fit into a microcentrifuge tube, it can be transferred to a 15 or 50 ml centrifuge tube and spun down with a larger centrifuge (see Note 18).
7. Carefully remove samples from the centrifuge and transfer the top aqueous layer to an RNase-free tube. Determine the exact volume of the aqueous layer and add 1.5 times the volume of 100% RNase-free ethanol and mix (see Note 19).
8. Filter the entire volume through an RNeasy microcolumn. For larger volumes (e.g., >5 ml), a vacuum manifold is suggested for faster sample processing (step 9a). Smaller volumes can be processed as individual 700 µl applications to the same RNeasy column and centrifuged (step 9b).

9a. *Vacuum manifold technique*: Using the QiaVac manifold (see Fig. 1) or equivalent (14), turn on the vacuum pump, and open the selected receiver ports to allow suction. Saturate receiver ports with RNaseZap for 30 s followed by rinsing with 100% ethanol. Turn off pump and aseptically install RNeasy microcolumn to selected receiver port(s). Turn vacuum pump on and repeatedly load 700 µl aliquots of the same sample into the RNeasy column until all of the sample volume has been filtered through. Remove column from vacuum manifold and place in a standard 2 ml capture tube and continue to step 10.

9b. *Centrifugation technique*: Apply no more than 700 µl to the RNeasy microcolumn and spin at >10,000 × *g* for 15 s. If the total volume is greater than 700 µl, multiple loadings to the same column will be required.

10. Perform DNase I treatment if required as per Subheading 3.3.1, step 7. If a DNase treatment is not needed, proceed to step 11 below.

11. Apply 700 µl RPE buffer to each column and centrifuge at >10,000 × *g* for 15 s. Discard and replace the waste capture tube containing the pass-through liquid.

12. Repeat step 11 a total of four times. This is required to remove any remaining Trizol that may otherwise be bound to the silica membrane when a Trizol-based lysis protocol is performed. Any residual Trizol contamination will lead to inaccurate UV-based RNA quantitation at 260 nm (see Note 19).

13. Using a 20 µl pipette, remove the remaining liquid that may be caught on the inner edge of the column's O-ring.

14. Perform an extended "dry" centrifugation at >10,000 × *g* for 2 min to remove as much residual liquid from the RNeasy microcolumn as possible. Do not centrifuge with column cap open.

15. Replace waste tube with a new standard RNase-free 1.5 ml microcentrifuge tube.

16. Apply 15 µl of 60°C RNase-free water directly to the center of the RNeasy microcolumn membrane, and incubate at room temperature for 30 s.

17. Centrifuge at >10,000 × *g* for 15 s.

18. Carefully remove the 15 µl of sample from the tube and reapply it to the same RNeasy membrane again. Close column

Fig. 1. Standard configuration for a vacuum manifold system fitted with RNeasy microcolumns. This approach allows the processing of large volumes of RNA extraction buffer (e.g., >5 ml) through the silica membrane without the use of a centrifuge.

and centrifuge at >10,000 × *g* for 15 s. This reelution is performed with the same 15 µl aliquot to assure complete recovery of RNA from the entire surface of the column.

19. Remove the RNeasy microcolumn from the microcentrifuge tube, and add the equivalent of 20 U of RNase inhibitor and vortex. At this point, samples may be stored at −20°C for short-term use or at −80°C for long-term storage.
20. Using both a UV spectrophotometer and fluorometer, such as the NanoDrop ND1000 and Qubit, determine the concentration of each sample. Make note of possible Trizol contamination as noted by a 270 nm absorbance peak on the UV spectrometer (see Subheading 3.4.1). In most cases, quantitative results for the fluorometer are lower than that of the UV spectrophotometer, but are considered more accurate.

3.3.2.2. Direct RNA Extraction Using RNeasy Microcolumn Method

When sorting directly into RLT buffer (guanidium isothiocyanate), a ratio of 100 µl of sorted sample to 350 µl of RLT should be maintained. In general, the Trizol LS method has a greater RNA recovery on cells with more resistant cell membranes, aggregated cells, or organisms with a cell wall, but is more costly and involves more reagents. RNA recovered by directly sorting into RLT buffer is typically much cleaner than that recovered with Trizol and does not require additional quantitation with a Qubit spectrofluorometer because there is no interfering 270 nm absorbance from trace amounts of Trizol carryover.

1. Start with 500 µl of RLT buffer with 5 µl BME in a sterile RNase-free FACS tube.
2. While sorting, periodically mix to get liquid off sides of the tube. Keep sample cold whenever possible.
3. After the sort, using a pipette with sterile tip, measure the final volume and calculate the exact volume of sample sorted into the RLT.
4. Adjust the amount of RLT required, so that a ratio of 350 µl of RLT buffer to every 100 µl of sorted sample is maintained and then vortex. Samples may need to be transferred to larger RNase-free tubes if final volumes are high.
5. Add 250 µl of 100% ethanol for every 350 µl RLT buffer, and then mix samples.
6. If volumes from step 5 are high (e.g., >5 ml), then use of a vacuum manifold is suggested. Smaller volumes can be processed as individual 700 µl applications to the same RNeasy microcolumn and centrifuged at >10,000 × *g* for 15 s.
7. Complete protocol by referring to steps 9a–19 in Subheading "Direct RNA Extraction Using a Combined Trizol LS-RNeasy Method".

3.4. Analyzing RNA from Sorted Melanoma Cell Fractions

3.4.1. Quantitation of RNA

After extracting RNA from $CD133^+$ and $CD133^-$ subsets using methods described above, the concentration of RNA was determined using a Nanodrop spectrophotometer and Qubit spectrofluorometer (15). Both methods are necessary because UV absorbance from the Nanodrop or other similar instruments alone cannot effectively discriminate some contaminants from true RNA; therefore, additional quantitation using a fluorescent RNA intercalation dye along with the Qubit spectrofluorometer is required (16). If residual Trizol carryover is present, an absorbance at 270 nm (Fig. 2) may be observed and interface with the absorbance value at 260 nm used for RNA and other nucleic acids resulting in erroneous quantification data. In cases where this carryover is problematic, further purification steps may be necessary. This may include an adjustment to the Trizol procedure to include an additional chloroform wash or a subsequent RNA cleanup step using a standard RNeasy MinElute column provided there is sufficient RNA available (see Note 19).

3.4.2. Assessing RNA Quality

RNA integrity was analyzed using the Agilent 2100 Bioanalyzer by loading 1 µl of sample RNA into the appropriate analysis cassette according to the manufacturer's protocol. For low RNA recovery samples (e.g., <10 ng/ul), the low-range Agilent Picochip cassette was required along with a 1 ng/µl RNA calibration ladder and three known RNA standards at a predetermined

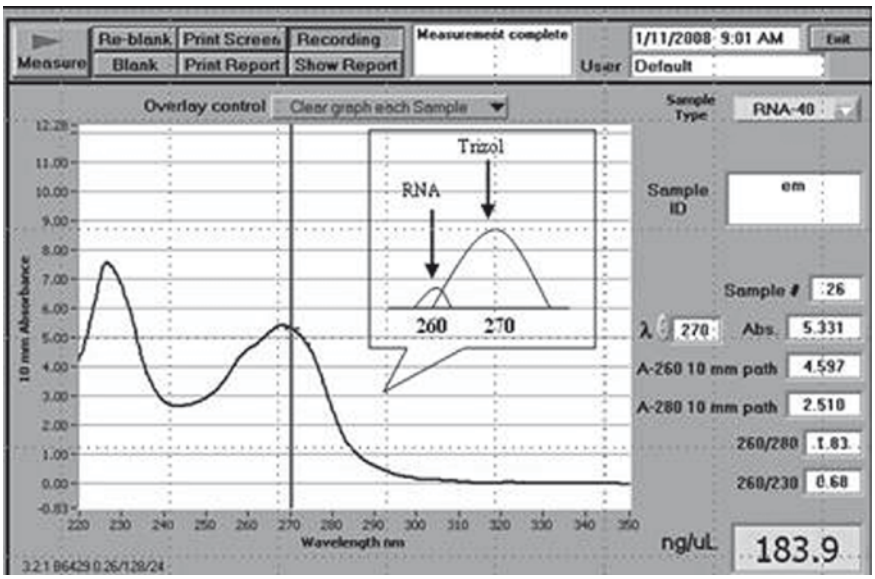

Fig. 2. Nanodrop ND-1000 trace of RNA recovered from FACS by a combined Trizol LS-RNeasy microcolumn strategy. Shown for illustrative purposes is the absorbance peak at 270 nm indicating the presence of residual Trizol contamination. This sample was further quantified using the Qubit spectrofluorometer, which uses the ribo-green nucleic acid dye to specifically intercalate RNA providing a more accurate RNA quantitation.

concentration of 1, 2, and 3 ng/μl to help empirically determine sample concentration (17). This method not only provides RNA integrity data but also provides quantitative data for samples that are below the effective range of the Nanodrop spectrophotometer. This was especially important for the rarer CD133$^+$ cell subset, where less than 10,000 cells were recovered.

Figure 3a shows high-quality RNA as indicated by the two sharp and well-defined ribosomal RNA peaks corresponding to the 18s and 28s subunits of total RNA recovered from the sorted CD133$^+$ melanoma cells. Figure 3b reveals RNA degradation as indicated by the reduction in the 28s subunit and appearance of smaller RNA fragments represented as smaller peaks to the left of the 18s signal. Figure 3c shows a separate sort from mouse liver CD45$^+$ cells with genomic DNA contamination illustrated by a "shoulder" between the 18s and 28s peaks. Figure 3d shows substantial removal of the genomic DNA after DNase I treatment.

3.4.3. Expected RNA Yields

We had previously determined RNA yield from various concentrations of cultured melanoma cells without FACS for later comparison of RNA yield after sorting. Presort RNA yields resulted in approximately 2–5 pg/cell. RNA extraction after FACS purification of CD133 melanoma subsets using the aforementioned protocols resulted in similar RNA yield, indicating comparable RNA

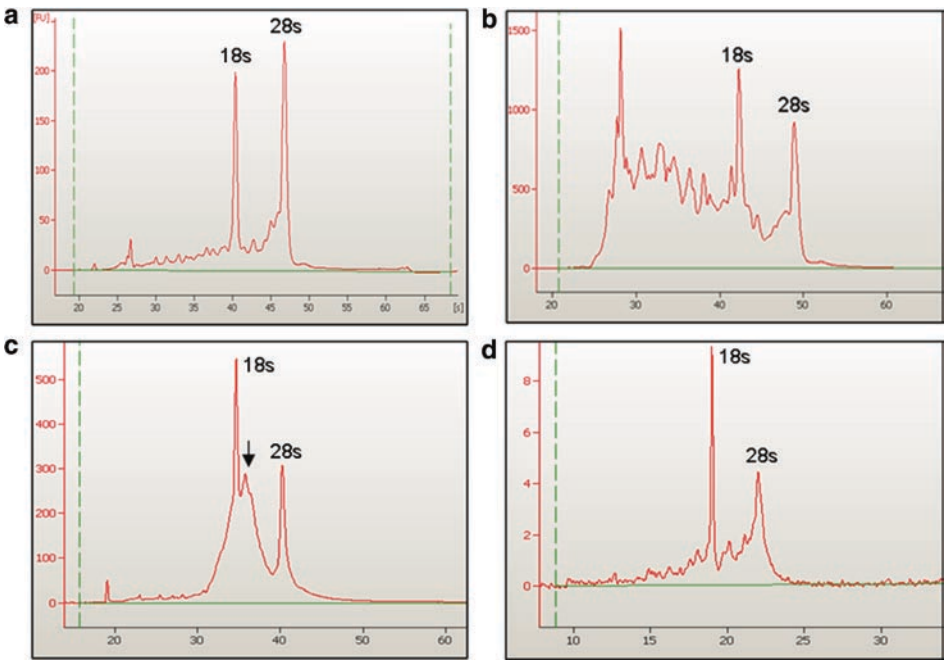

Fig. 3. RNA integrity profiles for FACS-purified cell samples using the Agilent 2100 Bioanalyzer. (**a**) Example of good quality RNA from purified mouse melanoma CD133$^+$ cells, (**b**) partially degraded RNA, (**c**) RNA contaminated with gDNA before and (**d**) after DNase treatment.

isolation efficiency when sorting cells with FACS. For comparison, we also sorted $CD45^+CD1d^+$ and $CD45^+CD1d^-$ mouse liver cells on the same BD FACSAria machine which resulted in similar dispensed sort volumes and RNA yields. Interestingly, a FACSCalibur mechanical sorter was used to sort human neuroblastoma cells and although dispensed sort volumes were far greater relative to the stream-in-air sorter, differences in RNA yield were unremarkable (see Table 1). While this data is not typically documented by most flow cytometry core facilities, its use for predictive sort volume recovery and quality control is vital and should be implemented as a routine performance indicator during each experiment.

4. Notes

1. During these procedures, it is assumed that all handling of tubes is performed using RNaseZap treatment for gloves and all surfaces, tubes, centrifuges, and pipettes. Flame sterilization should be adopted for any utensils where applicable. It is also recommended to perform all steps in a low-travel area and biosafety cabinet or PCR hood. At no point should RNaseZap be used in place of an RNase inhibitor. These types of surface decontamination solutions are composed of alkali hydroxides or chlorine derivatives such as sodium dichlorocyanurates and if exposed to samples will kill cells and degrade RNA; therefore, they are intended for sanitizing solid surfaces and utensils only.
2. Before sorting critical cells for RNA isolation, perform a FACS instrument QC test run using one test sample with a known viability greater than 80%. This sample is divided into two tubes: (1) a no sort control and (2) a postsort control. Both samples should be extracted for RNA simultaneously and analyzed for RNA integrity on the Agilent 2100 bioanalyzer. If both test samples result in degraded RNA (see Subheading 3.4.2), a review of handling and reagents is necessary. If RNA quality is good for the no sort control only, then RNase contamination of the flow cytometer is likely. Reagents and sheath fluids can be tested using such reagents as RNaseAlert (or equivalent) available from several vendors.
3. Fixation of cells using formalin and other aldehydes should be avoided because it causes nucleic acid cross-linking and contributes to RNA degradation. Although ethanol fixation does not negatively affect RNA, it does cause cell membrane permeability and possible mRNA leakage. For these reasons, it is preferable to use cell surface markers in order to avoid cell

Table 1
Summary of FACS data for RNA isolated from mouse melanoma CD133$^+$ and CD133$^-$ cell fractions sorted using a BD FACSAria compared to normal mouse liver CD45$^+$CD1d$^-$ and CD45$^+$CD1d$^+$ cells and human neuroblastoma cells sorted using either a BD FACSAria or BD FACSCalibur, respectively

Cell type	Sorter type	RNA isolation method	Nozzle size (μm)	#Cells sorted	Targeted subset	Sorted volume	nl/sorted cell	RNA (ng)	pg RNA/cell
Mouse melanoma	Stream-in-air sorter	Subheading "Direct RNA extraction using RNeasy micro-column method"	100	1.0×10^5	CD133–	550 µl	5.5	250	2.5
				5.0×10^3	CD133+	30 µl	6	16	3.2
				1.0×10^5	CD133–	550 µl	5.5	270	2.7
				7.5×10^3	CD133+	45 µl	6	28	3.7
				5.0×10^4	CD133–	550 µl	11	145	2.9
				1.0×10^4	CD133+	60 µl	6	40	4
Mouse liver	Stream-in-air sorter	Subheading "Direct RNA extraction using a combined Trizol LS-RNeasy method"	100	1.0×10^6	CD45+ CD1d–	7.8 ml	7.7	408	0.41
				3.0×10^5	CD45+ CD1d+	2.8 ml	9.1	172	0.57
				1.7×10^6	CD45+ CD1d–	13.6 ml	8	900	0.53
				5.7×10^5	CD45+ CD1d+	4.5 ml	7.8	322	0.56
Human neuro-blastoma	Mechanical sorter	Subheading 3.3.1	Catcher tube	1.4×10^4	n/a	90 ml	690	44	3.3
				1.2×10^4	n/a	90 ml	740	19	1.6
				2.0×10^4	n/a	90 ml	450	64	3.2
				5.5×10^4	n/a	90 ml	160	44	0.44

fixation–permeabilization before FACS for optimal RNA yield and integrity.

4. The use of RNase-free BSA (acetylated BSA) in place of serum in sample buffers is preferred as some serum sources may be inherently contaminated with RNases.
5. Samples with viability less then 80% viability are likely to result in partially degraded RNA and may benefit from the addition of 100 U of RNase inhibitor/ml of sample before performing cell sorts.
6. When sorting into precooled sterile RNase-free FACS tubes, it is important to consider adding an RNase inhibitor prior to initiating the sort. During the sort, the sample should be periodically mixed.
7. The physical stresses of FACS and centrifugation may lead to artifactual gene expression pattern changes of the sorted cells and it is recommended to work quickly when centrifuging cells after the sort and lysing cells with the RNA isolation buffer immediately following the centrifugation while keeping samples cold. Additionally, sort all samples in an experiment using the same reagents, cytometer settings, centrifugation forces, and temperatures throughout the procedure.
8. The total dispensed volume can be predicted beforehand by taking the target number of cells desired for sorting and multiplying by the approximate volume of each cell droplet. For example, if using an electrostatic sorter with a 100 μm nozzle, and it is wished to sort 10^5 cells, then the total dispensed liquid during the FACS run will be between 5 nl $\times 10^5$ and 10 nl $\times 10^5$, or 500 μl to 1 ml. However, empirical testing to determine the exact dispensed FACS volumes is recommended.
9. Alternative silica columns, such as RNeasy Mini, Invitrogen's PureLink RNA Micro kit, and Ambion's RNAqueous microcolumns, have similar silica technology and are amendable to the procedures outlined above with minor changes. Magnetic bead RNA isolation procedures (e.g., Dynabeads® from Dynal Corp. or MACS® from Miltenyi Biotec) have also been adapted for isolating RNA from FACS samples and recent data suggest improved RNA recoveries over those observed with standard silica-based columns (7). However, large volume extractions have yet to be investigated.
10. Unless small RNA species are required from the sorted cells, a silica column-based approach is advantageous due to its capacity for recovering small amounts of RNA from a limited number of cells. Recovery of small RNA species, such as miRNAs, is best accomplished using a standard Trizol–chloroform

precipitation procedure which involves centrifuging the sorted cells to a pellet and extracting using the manufacturer's recommended protocol (12). However, when small numbers of cells are expected, the precipitation must be performed using a nucleic acid coprecipitate such as PelletPaint or GlycoBlue in an Axygen MaxyClear MCT175C centrifuge tube for maximum RNA pellet formation.

11. Before using an RNase inhibitor, it is necessary to review downstream applications of the RNA because some methods cannot tolerate the presence of an RNase inhibitor. Understandably, when sort volumes are high (>25 ml), it is not economical to maintain a final RNase inhibitor concentration at 20 U/ml. Regardless of the final concentration selection, it is most important to maintain consistency for samples belonging to the same experiment.
12. During a sort, it is not recommended to sort into RNA*later* or other ammonium sulfate solutions as the resulting viscosity will be too high to centrifuge the cells properly and result in poor cellular recovery and compromised RNA quality. This is not unexpected as this reagent is designed for tissue preservation and not for purified cells from FACS (18).
13. A DNase I treatment will be required when downstream methods involving random hexamer priming such as in the case of exon microarrays, RT-qPCR, or equivalent. If no DNase I treatment is required, it should be omitted as results from our laboratory indicate that an expected loss of 30–40% of RNA may be observed when performing an on-column digestion (unpublished data).
14. Freezing directly sorted extracts in Trizol or RLT buffer often results in degraded RNA and is not recommended. However, we have observed that samples that are maintained at 4°C overnight in a dilute (~20%) Trizol LS solution followed by proper RNA extraction the next day have resulted in good quality RNA. Any storage method should be evaluated on each sample type prior to beginning an experiment because some cell types do not tolerate any lengthy Trizol or RLT exposure.
15. Although direct sorting of cells into RNA extraction buffer will negate a postsort cell purity check, this can still be performed by separately sorting a fraction of the cells into another tube containing PBS with 2% BSA, so that purity analysis can be performed after FACS is complete.
16. The example below indicates the amount of each reagent required to process a sample from the method outlined in Subheading 3.3.2. In this example, the cell lysis, nucleic acid

separation, and ethanol steps must be done in either a 15 or 50 ml RNase-free centrifuge tube and a vacuum manifold will be needed for processing the RNeasy microcolumn:

Original Trizol LS in FACS Tube (presort)	0.5 ml
Sorted volume (Total volume postsort minus 0.5 ml Trizol LS above)	2.2 ml
Trizol LS needed to maintain 3:1 ratio (Trizol:sample ratio, 6.6:2.2)	6.1 ml
Amount of chloroform needed (0.2 ml/0.75 ml Trizol LS)	1.8 ml
Total volume for centrifugation	10.6 ml
Recovered aqueous phase (AQP)	4.5 ml
100% ethanol needed (1.5 × AQP v/v)	6.8 ml
Total volume to be applied to column	11.3 ml

17. The use of alternative organic phases in Trizol precipitations, such as 1-bromo-3-chloropropane (BCP) and 4-bromoanisole (4BA), has proven to be less desirable in our facility as their vapor pressures are low and do not benefit by evaporating from the final sample such as in the case of chloroform.
18. When using larger centrifuge tubes to processing larger volumes of the Trizol sample mix, it is not possible to centrifuge at 12,000 × *g*, and we have found that spinning as low as 1,000 × *g* results in good quality RNA.
19. Unfortunately, the Nanodrop and Qubit instruments cannot effectively discriminate RNA from DNA and other 260 nm absorbing contaminants. Any resulting DNA contamination must either be characterized or digested before proceeding to downstream reactions. Trizol carryover (absorbance at 270 nm) can sometimes be minimized by adding an additional chloroform cleanup step. This is done by combining the recovered aqueous phase with an equal volume of fresh chloroform at step 7 in Subheading "Direct RNA Extraction Using a Combined Trizol LS-RNeasy Method". The tube is mixed and incubated at room temperature before centrifuging at full speed. The resulting aqueous phase is then processed exactly as the original aqueous phase at step 7 of Subheading "Direct RNA Extraction Using a Combined Trizol LS-RNeasy Method" by combining with a 1.5× volume of ethanol.

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