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Analyzing the Circulating MicroRNAs in Exosomes/Extracellular Vesicles from Serum or Plasma by qRT-PCR

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Abstract

Small extracellular vesicles are released from both healthy and disease cells to facilitate cellular communication. They have a wide variety of names including exosomes, microvesicles and microparticles. Depending on their size, very small extracellular vesicles originating from the endocytic pathway have been called exosomes and in some cases nanovesicles. Collectively, extracellular vesicles are important mediators of a wide variety of functions including immune cell development and homeostasis. Encapsulated in the extracellular vesicles are proteins and nucleic acids including mRNA and microRNA molecules. MicroRNAs are small, non-coding RNA molecules implicated in the post-transcriptional control of gene expression that have emerged as important regulatory molecules and are involved in disease pathogenesis including cancer. In some diseases, not only does the quantity and the subpopulations of extracellular vesicles change in the peripheral blood but also microRNAs. Here, we described the analysis of peripheral blood extracellular vesicles by flow cytometry and the RNA extraction from extracellular vesicles isolated from the plasma or serum to profile microRNA expression.

Keywords

microRNA; Extracellular vesicles; Quantitative reverse transcription real-time PCR (qRT-PCR); Flow cytometry; RNA extraction

1 Introduction

Previously, we characterized microRNA expression in extracellular vesicles from the peripheral blood of normal healthy individuals [1]. Using flow cytometric staining for surface antigens, we and others have found that extracellular vesicles in the peripheral blood largely originate from platelets [1–3], while the myeloid-derived extracellular vesicles constitute a large component of the peripheral blood extracellular vesicles [1]. Changes in extracellular vesicles quantity and subpopulations occur in a variety disease states including cancer and severe sepsis [2–12]. Importantly, the microRNA pattern is also altered in these diseases and may reflect early disease diagnosis and progression. Thus, not only the plasma extracellular vesicles but also the microRNAs contained in the extracellular vesicles may serve as biomarkers of health and disease.

Here, we will describe in detail our method for purifying and characterization of extracellular vesicles from the peripheral blood. Many investigators have reported the utilization of differential high speed centrifugation to isolate small exosomes (<200 nm) and larger extracellular vesicles (200–1,000 nm) [1, 4, 7, 9, 12, 13]. Using flow cytometry and polystyrene beads of various sizes (70–2,000 nm), the quantity of extracellular vesicles can be determined from plasma or serum, as previously described [14]. Furthermore, since the

extracellular vesicles are membrane-bound and contain surface antigens from their cellular source, one can easily use flow cytometric analysis with some modifications to determine their origin by immunofluorescence staining [1].

Previously, we have reported the microRNA signature for over 480 known microRNAs from the plasma [1]. Our initial study required a large volume of blood to isolate plasma microRNAs from peripheral blood extracellular vesicles. Since blood volumes are often limiting in the elderly, seriously ill or children, we have since optimized the RNA extraction procedure for small volumes. From this optimized protocol, we can extract sufficient RNA to profile a large number of microRNAs by either qRT-PCR or microarrays.

2 Materials

2.1 Blood Collection Components

1. EDTA vacutainers.
2. P100 plasma separator tubes.
3. Serum separator tubes.

2.2 Extracellular Vesicle Purification Components

1. RNase/DNase free microcentrifuge tubes.
2. Beckman JLA 30.50 Ti rotor.
3. Nalgene Oak Ridge conical tubes, sealing caps and tube adapter.

2.3 Flow Cytometry Components and Solutions

1. PBS (Phosphate Buffered Saline)/NaN₃ (Sodium Azide)/BSA (Bovine serum albumin) solution containing PBS, 0.1 % w/v NaN₃ and 1 % w/v BSA.
2. Polystyrene calibration beads: 2.07 µM, 1.09 µM, 0.2 µM and 0.07 µM. Make a 1:1,000 dilution for each polystyrene bead suspension in PBS/NaN₃/BSA solution. After dilution, the concentrations should be: 10,000 beads/ µL for 2.1 µm bead; 70,000 beads/ µL for 1.09 µm beads; 11,000,000 beads/µL for 0.2 µm beads; and 260,000,000 beads/µL for 0.07 µm beads.
3. Human IgG. Extracellular vesicles will be blocked with 100 µg/mL human IgG solution (1 µL of 11.3 mg/mL stock)
4. Fluorescently-conjugated FAB' antibodies to cell surface antigens and isotype control antibodies.
5. Annexin V-FITC.
6. Syto RNASelect Green Fluorescent dye (Life Technologies) (*see* Note 1).
7. Hoechst 33342 DNA Stain. Working concentration: 0.2–0.5 µg/mL.
8. Optional: 4 % Paraformaldehyde Solution. Weigh 100 mL bottle and cap. In chemical fume hood, add paraformaldehyde to bottle and recap. Weigh capped bottle with paraformaldehyde. In chemical fume hood, add PBS to achieve a 4 % w/v solution, 2–3 pellets of NaOH (Sodium hydroxide), and stir bar. Place capped

¹Our laboratory has found that the Syto RNASelect Green Fluorescent dye supplied by Life Technologies works well for staining RNA molecules contained in the extracellular vesicles to visualize by flow cytometry. While other RNA binding dyes may work in this capacity, the investigator should empirically evaluate the concentration of these dyes and ability to observe the fluorescently labeled extracellular vesicles by flow cytometry in their own laboratory.

bottle on stir plate. When paraformaldehyde is in solution, it is safe to open the bottle outside of fume hood. Adjust pH to 7.1–7.3. Store solution for 1–6 months at 4°C (*see* Note 2).

9. BD LSR II flow cytometer (BD Biosciences), configured with three lasers; 488 nm, 405 nm and 633 nm) with the ability to detect up to 13 colors, including three channels for Qdots (*see* Note 3).

2.4 RNA Extraction Components

1. Molecular grade chloroform, water and ethyl alcohol.
2. The miRNeasy Mini Kit (Qiagen) (*see* Note 4).
3. RNase/DNase free 2 mL safe-lock microfuge tubes.
4. Synthetic *C. elegans* microRNAs: Syn-cel-miR-39, ID # MSY0000010 (UCACCGGGUGUAAAUCAGCUUG), Syn-cel-miR-54 ID # MSY0000025 (UACCCGUAUUCUUAUUAUCCGAG) and Syn-cel-miR-238, ID # MSY0000293 (UACCCGUAUUCUUAUUAUCCGAG). Diluted to 25 nM each in molecular grade water and store aliquoted at –20 °C.

2.5 cDNA and Quantitative Real-Time PCR (qRT-PCR) Profiling Reagents and Solutions

1. Recombinant DNase I, RNase free.
2. Human Ribonuclease Inhibitor.
3. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Carlsbad, CA) (*see* Note 5).
4. Using the High Capacity cDNA Reverse Transcription Kit prepare a Transcription Master Mix containing: 0.25 µL dNTPs, 5 µL 10× RT buffer, 6 µL 25 mM MgCl₂, 0.5 µL RNase inhibitor, 10 µL Multiscribe™ reverse transcriptase and 3.25 µL molecular grade water, for each cDNA sample.
5. TaqMan® MicroRNA Assay, Mature Human MicroRNAs (Applied Biosystems), purchased as Human Pool A v2.1, Human Pool B v3.0 or a Custom Human Pool. MicroRNA assays for endogenous controls should also be purchased separately if not in the pools. These include Human 18S, RNU43, RNU38B and U6 as well as assays for the three exogenous controls to *C. elegans* microRNAs; cel-miRs-39, -54, and -238.
6. Optional: Megaplex™ PreAmp Primers, Human Pool A v2.1 (Applied Biosystems).
7. Optional: Megaplex™ PreAmp Primers, Human Pool B v3.0 (Applied Biosystems).
8. Optional: TaqMan® PreAmp Master Mix (Applied Biosystems).

²Paraformaldehyde powder is toxic and flammable. It is important to use a chemical hood and follow instructions when preparing the solution.

³While we use the BD LSR II flow cytometer, Jayachandran *et al.* recently reported better resolution of the smaller polystyrene beads with the FACSCanto II cytometer (BD Biosciences) [17]. Thus, this cytometer may be better to quantitate extracellular vesicles.

⁴Here, we present an optimized protocol using the Qiagen kit to isolate RNA directly from plasma or serum without pelleting the extracellular vesicles.

⁵We [1] and colleagues [16] have optimized the qRT-PCR profiling method using reagents from Applied Biosystems, Life Technologies, Carlsbad, CA. It is possible that the Qiagen SYBR® green-based *miScript* PCR System, which enables the profiling of several hundred miRNAs from the same sample may be used. However, we have not investigated this kit with the parameters outlined.

9. TaqMan® Universal Master Mix, No AmpErase® UNG (Applied Biosystems).
10. V-bottom, 96-well plate with adhesive seals.
11. 384-well PCR plates with optical adhesive film.
12. Sterile RNase/DNase free basin.
13. Freezer storage bags.
14. Biomek® FX Liquid Handling System (Beckman Coulter, Inc., Fullerton, CA), manual or electronic 96-channel pipetting station or a 12-channel pipette/repeating pipette.
15. 7900 HT Fast Real-Time PCR System with 384-well block module (Applied Biosystems).

3 Methods

3.1 Blood Collection and Processing

1. Following informed consent, collect blood to isolate either plasma using P100 or EDTA vacutainers or serum in a serum separator (*see* Note 6).
2. All blood samples except the P100 samples should be processed within 1 h from collection. Samples in P100 tubes can be processed within 1–24 h from collection. If not processing immediately, P100 samples should be kept at room temperature.
3. Centrifuge the tubes as follows:
 - a. EDTA anti-coagulated blood tubes should be centrifuged at $1,100 \times g$ for 20 min at room temperature.
 - b. Follow the manufacturer instructions to clot the blood in the Serum separator tubes. The tubes are then centrifuged at $1,100 \times g$ for 15 min at room temperature.
 - c. For the P100 collection tubes, the samples should be centrifuged at $2,500 \times g$ for 20 min at room temperature.
4. Plasma and serum are either used immediately or stored at -80°C for various amounts of time.

3.2 Quantification of Total Extracellular Vesicles

1. To remove platelets, centrifuge plasma at $4,500 \times g$, 15 min, at 10°C . This step can be repeated to remove any additional platelets that may remain.
2. Aliquot platelet-poor plasma or serum in 1.5 mL microcentrifuge tubes. Use the platelet-poor plasma or serum directly or concentrate extracellular vesicles by centrifugation at $16,500\text{--}21,000 \times g$ for 30 min at 4°C .
3. Alternate optional procedure: Recent studies report non-vesicle bound RNA molecules in circulation [15]. Based on unpublished data from our laboratory, enhanced RNA isolation can be accomplished by using $160,000 \times g$ centrifugation. While the vesicle distribution of large ($>200\text{ nm}$) and smaller extracellular vesicles including exosomes ($<200\text{ nm}$) is similar between the $16,500$ and $21,000 \times g$ and

⁶When RNA isolated from the extracellular vesicles is to be used for qRT-PCR avoid blood collection tubes containing citrate or heparin. Both anticoagulants interfere with PCR (unpublished data) [18–20]. Heparin interferes with enzymatic activity of polymerase [18, 19], while citrate binds DNA [20].

160,000 $\times g$ centrifugations [21], we currently do not know if this enhancement is due to increased vesicles in the pellet or the non-vesicle RNA concentration. The sample from **Step 2** is placed in a Nalgene oak ridge conical tube and centrifuged at 160,000 $\times g$ for 90 min at 4 °C using Beckman JLA 30.50 Ti rotor. For smaller sample sizes (<1 mL), the supernatant can be transferred to 11 $\times$ 34 mm polycarbonate Beckman centrifuge tubes (cat # 343778, Beckman Coulter, Brea, CA) and centrifuged at the same settings as described using a Beckman TLA 120.2 rotor.

4. Resuspend the concentrated extracellular vesicle pellets in 500 μ L of PBS/ NaN₃/ BSA solution (*see* Note 7).
5. To 500 μ L of plasma or serum containing extracellular vesicles or concentrated extracellular vesicles, add a known quantity of 2.1 μ m beads. We prefer to add 20,000 beads at a concentration of 10,000/ μ L, which is 2 μ L of diluted beads (*see* Note 8).
6. The other bead standards are used for qualitative analysis of size. Add 2 μ L of each bead size alone to 500 μ L of PBS/Azide/BSA solution.
7. Optional: Incorporation of flow cytometric staining and exclusion of apoptotic bodies (*see* below, Subheadings 3.3 and 3.4, respectively).
8. Cytometer settings vary depending on manufacturer and sensitivity desired. However for the BD LSR II flow cytometer, reduce the default threshold of 5,000–1,000 and 200 for FSC and SSC, respectively (*see* Note 9). Make sure FSC and SSC are set at log scale.
9. Run the bead only sample first (*see* Notes 10 and ¹¹). Set gates around each different bead size (Fig. 1). Create another gate that encompasses beads 0.07–1.09 μ m and excludes 2.1 μ m beads, designated Gate 4. Lastly, create Gate 5 that encompasses only 0.2 and 1.09 μ m beads.
10. Set the data acquisition to stop when the bead count in the 2.1 μ m bead region reaches 1,000 events and run each sample (Fig. 1).
11. To calculate the extracellular vesicles concentration (n), (*see* Note 12).

$$a = \text{Sample Events Gate 5} - \text{Background (Media only Events in Gate 5)}$$

$$b = a \times (\text{Beads Added/Beads Counted})$$

$$n = b \times \text{dilution factor} \times \text{sample volume}$$

3.3 Fluorescent Surface Antigen Staining of Extracellular Vesicles

1. For blocking to reduce non-specific antibody binding, add 5 μ L of IgG per sample and incubate on ice for 10–30 min.
2. Add desired antibody or isotype control antibody to the samples. Check data sheets for recommended antibody usage for one million cells (*see* Note 13).

⁷A minimum volume of at least 300 μ L is required for the flow cytometer.

⁸Vortex the beads well before adding to the sample or PBS/Azide/BSA solution.

⁹It may be difficult to adequately resolve the 70 nm beads on the BD LSR II flow cytometer due to noise from the cytometer (*see* Note 3).

¹⁰Since there is noise when one reduces the threshold, it is important to have a medium or sample only for a background control.

¹¹To ensure beads are adequately mixed in the samples, vortex all samples prior to running on the flow cytometer.

¹²Sample calculations of extracellular vesicles using 500 μ L from 5 mL of plasma with no dilution and $a = 4,500$. $b = 4,500 \times (20,000/1,000) = 90,000$ $n = 90,000 \times 1 \times 5 \text{ mL} = 450,000$ total extracellular vesicles

3. Incubate on ice protected from light for 30 min to 1 h.
4. Analyze on flow cytometer immediately using settings described above (Subheading 3.2, *see* Step 8).
5. If not analyzing immediately, add 250 μ L of 4 % paraformaldehyde and store samples in the dark at 4 °C for up to 5 days.

3.4 Analysis of Nucleic Acid Content and Annexin V Expression on Extracellular Vesicles

1. To determine the presence of apoptotic bodies by DNA content and Annexin V expression, extracellular vesicles are incubated with 4.86 μ M Hoechst 33342 and 2.5 μ L of Annexin V-FITC for 30 min on ice [21]. Protect from light during incubation.
2. A separate extracellular vesicles sample is incubated with 0.625 μ M SytoRNA for 30 min on ice. Similarly the samples are protected from light during the incubation.
3. The stained samples are then washed twice by centrifugation at 16,500–21,000 $\times g$ for 30 min at 4 °C. The extracellular vesicle pellet is then resuspended in 500 μ L of PBS/Azide/BSA solution.
4. Using the FSC and SSC settings described above (Subheading 3.2, *see* Step 8), RNA content is analyzed on BD LSR II flow cytometer using the laser that excites at 488 nm. Data is then presented as FITC (FL1) vs. FSC. The DNA content (Hoechst 33342) and Annexin V-FITC are excited with the excitation lasers at 405 and 488 nm, respectively. Fluorescence is plotted as Hoechst 33342 vs. Annexin V-FITC.

3.5 RNA Extraction Directly from Plasma or Serum Extracellular Vesicles

1. To 250 μ L of plasma or serum in a 2 mL safe-lock tube, add 1.5 mL of QIAzol Lysis Reagent; vortex well or pipet to mix.
2. Incubate the tube containing the homogenate at room temperature (15–25 °C) for 5 min to promote dissociation of nucleoprotein complexes.
3. Add 25 fmol *C. elegans* microRNA mixture in a total of 5 μ L to each sample. These will be used as exogenous controls during the PCR procedures.
4. Add 250 μ L of chloroform to the tube containing the homogenate and *C. elegans* microRNAs then vortex the tube for 15 s.
5. Incubate the tube at room temperature 2–3 min.
6. Centrifuge the tube for 15 min at 12,000 $\times g$, 4 °C.
7. The volume of the aqueous phase will be approximately 1 mL. Transfer 350 μ L aliquots of the upper aqueous phase to 2 safelock microfuge tubes (*see* Note 14). Set aside for QIAcube extraction (Subheading 3.6).
8. Transfer the last 350 μ L into a 1.5 mL tube and add 525 μ L (1.5 vol) of 100 % ethanol. Mix thoroughly by pipetting up and down several times. Do not centrifuge.
9. Distribute equally the ~875 μ L sample on two miRNeasy Mini spin columns and centrifuge for 15 s at 12,000 $\times g$, at room temperature (*see* Note 15). Discard the

¹³Include an isotype control for each fluorescent antibody. If possible, consider purchasing Fab/fluorescent antibodies to reduce nonspecific binding.

¹⁴At this step, care should be taken not to perturb the interface and contaminate the extract with either protein precipitate or phenol.

¹⁵Wherever the Qiagen miRNeasy protocol recommended 15 s at 8,000 $\times g$, we modified the centrifugation to 12,000 $\times g$ for 15 s.

flow-through. At this point, one can either use the QIAcube Procedure (Subheading 3.6) or if a QIAcube is unavailable then proceed to the Manual Extraction Protocol (Subheading 3.7).

3.6 QIAcube Procedure

1. Place both minicolumns and the two 350 μL safe-lock tubes from **Steps 7 to 9** (Subheading 3.5) containing the aqueous phase in the QIAcube instrument. Make certain tubes, minicolumns and buffers are in the proper slots, as indicated in the instrument protocol and according to the number of samples.
2. Start the protocol “miRNeasy aqueous phase.” Follow the instrument instructions, and when prompted set the final elution volume to 30 μL (*see* Note 16).
3. Pool the two 30 μL aliquots from the minicolumns and determine the RNA concentration using Nanodrop or equivalent.

3.7 Optional: Manual Extraction Protocol

1. When a Qiacube is unavailable follow miRNeasy procedure, using two minicolumns, rather than one per sample, by dividing the aqueous phase plus ethanol onto two minicolumns. Wash each RNeasy Mini spin column by adding 700 μL of RWT buffer and centrifuge for 15 s at $12,000 \times g$. Discard the flow-through.
2. Then add 500 μL of RPE buffer to the RNeasy Mini spin column and repeat the $12,000 \times g$ centrifugation for 15 s. Again discard the flow-through from this wash step.
3. Repeat the wash step of the RNeasy Mini spin column with another 500 μL of RPE buffer. To ensure all buffer is removed from the column, centrifuge for 2 min $12,000 \times g$ and discard the flow-through.
4. To ensure the column is completely dry before eluting the RNA, discard the old collection tube with the flow-through and place the RNeasy Mini spin column into a new collection tube. Centrifuge the column at full speed for 1 min in the microcentrifuge (*see* Note 17).
5. Transfer the RNeasy Mini spin column to a new 1.5 mL collection tube.
6. Add 30 μL of RNase-free water directly onto the RNeasy Mini spin column membrane, wait 1 min.
7. To elute the RNA, centrifuge the RNeasy Mini spin column for 2 min at $12,000 \times g$. For each sample, pool the 30 μL of eluates from the multiple spin columns and measure microRNA concentration.

3.8 Ordering, Arrangement and Plating TaqMan® microRNA PCR Primers in 384-Well Plates

1. To profile a large number of microRNAs simultaneously, a 384-well qRT-PCR platform is strongly encouraged [16].
2. Applied Biosystems (Life Technologies) segregates a vast majority of the mature microRNA assays into two groups designated as Human Pool A v2.1 and Human

¹⁶Since the samples will be quite dilute (in the range of 10–25 ng/ μL), we recommend the minimum elution volume, which is 30 μL . Due to decrease RNA recovery from the columns, it is not recommended to use elution volumes less than 30 μL .

¹⁷The Qiagen protocol mentions this step as “Optional,” however high quality RNA is obtained by its inclusion.

Pool B v3.0. Of all the currently 1,588 mature assays available through Applied Biosystems, the Human Pool A v2.1 and Human Pool B v3.0 comprises of 375 and 354 mature microRNA assays, respectively. Of the remaining microRNA assays not encompassed by these pools, we have selected approximately 274 additional assays, that we designate Pool C (*see* Note 18). Notably, all microRNA pools contain control assays for U6, RNU43, RNU38B and cel-miRs-39, -54, and -238.

3. The 20× TaqMan[®] PCR primers for each pool are shipped in 96-slot boxes. Thus, for Human Pool A v2.1 and Human Pool B v3.0, there are four 96-slot boxes per pool. This serves as an advantage to arrange the TaqMan[®] PCR primer vials to 384-well format and simplifies the plating process.
4. To easily arrange vials for plating in the 384-well format, we have generated a template that takes the 384-well format (24 columns × 16 rows) and divides the plate in 2 × 2 quadrants. Ultimately, this generates a template with 12 quadrants across the plate × 8 quadrants down the plate corresponding to a 96 well format (*see* Note 19, Fig. 2) and designated as quadrants A1–A12, B1–B12, etc. The top left of each quadrant corresponds to the first 96-slot box of TaqMan[®] PCR primer pool while the top right of each quadrant corresponds to the second 96-slot box of the primer pool as designated by Box 1 and 2, respectively. As such, the bottom left and bottom right corresponds to the third and fourth 96-slot box TaqMan[®] PCR primer pool. The A1 quadrant will be plated with TaqMan[®] PCR primers corresponding to the A1 position of each 96-slot box from the primer pool and so forth.
5. It is very important to include endogenous controls on each plate. The controls can vary among plates and ideally it is recommended to have some repeated between the 384-well plates. Controls should include 18S, RNU43, RNU38B and U6 as well as exogenous controls to the three *C. elegans* microRNAs (Subheading 3.5, **Step 5**).
6. Each 384-well plate represents a single replicate. Therefore, to run a sample in duplicate, two 384 well plates are required per microRNA assay pool.
7. During the arrangement of microRNAs for plating, keep all 96-slot boxes on dry ice. Store primers at –20 °C until needed for plating (*see* Note 20).
8. Once a template is drawn out with the arranged microRNA assays, a template file is created in Applied Biosystems' Sequence Detection System (SDS) software v2.2.3 for each of the three pools (*see* Note 15).
9. To each well of the 384-well plate, 10 µL of diluted TaqMan[®] PCR primers is required. Since two 384-well plates are required to run a sample in duplicate, a minimum of 22 µL of each TaqMan[®] PCR primer is required to account for residual volumes in pipette tips (*see* Notes 21 and ²²).

¹⁸Previously, we used the 931 mature, human TaqMan[®] MicroRNA assay sets [1] which is no longer available through Applied Biosystems. In order to have a similar coverage, the microRNA assays from this original 931 mature human TaqMan[®] MicroRNA assay sets not found in the Human Pool A v2.1 and Human Pool B v3.0 are ordered separately and designated Pool C. We have also included several interesting microRNAs for a total of 274 microRNA assays in this pool. Of particular note, to order microRNAs not contained in the two pools, the purchaser must assemble a text file listing the assay ID numbers for all microRNAs to be purchased.

¹⁹Using the template in Fig. 2, creating a map of the microRNA assays and their positions in the storage boxes/racks and 384-well plates is extremely helpful. Also, in order to minimize time outside of the freezer, it is recommended that all desired layouts be determined and double-checked prior to arranging primer vials.

²⁰When handling TaqMan[®] primers, care should be taken to avoid prolonged exposure to light whenever possible, due to the fluorescent probe.

²¹To compensate for loss of volume during pipetting, make an extra 10 % of all solutions including DNase, Reverse Transcription, RT-PCR and Pre-Amplification Master Mixes as well as primers.

10. Thaw on ice the TaqMan[®] PCR primers in each 96-slot box and pulse spin primers from the desired pool(s) in their 96-slot box.
11. Dilute the TaqMan[®] PCR primers from each 96-slot box of TaqMan[®] PCR primers 1:40 in a V-bottom 96-well plate using molecular grade water.
12. Prepare each V-bottom 96-well plate with the required molecular grade water and labeled with appropriate pool name and box number (*see* Note 23).
13. While on ice, uncap vials one row at a time and using a multi-channel pipette transfer the necessary volume of TaqMan[®] PCR primer to the appropriate wells in the 96-well plates. Recap vials and promptly return primers to -20°C when finished.
14. When the TaqMan[®] PCR primers are diluted, seal the 96-well plates with adhesive lid and store at 4°C in the dark.
15. To dispense the diluted TaqMan[®] PCR primers in the 384-well plates, it is recommended that an automated liquid handling system is used with the provided template (*see* Note 19, Fig. 2). If a robotic platform is not available, a manual or electronic 96-channel pipetting station or a 12-channel pipette/repeating pipette may be used instead.
16. From the 96-well plates, 10 μL of diluted primer is transferred to the appropriate wells in the 384-well plate (Fig. 2).
17. The 384-well plates are allowed to completely dry at room temperature in a clean ventilated hood area and away from direct light. The desired result is to evaporate the entire 10 μL volume. This generally requires an overnight incubation.
18. Bag the dried plates, 6 plates per freezer storage bag. Do not stack. Store plates at -20°C until needed for qRT-PCR. When stored properly, the plates are good for 3–4 months after plating.

3.9 Reverse Transcription (RT) for microRNA Analysis

1. To simplify cDNA production and conserve primers, we perform a pooled reverse transcription (RT) reaction that corresponds to each TaqMan[®] PCR Primers Pools (Subheading 3.8) [16].
2. Combine 10 μL of the supplied $5 \times$ Reverse Transcription (RT) Primers for each pool in a sterile RNase/DNase free basin. Pool A and Pool B, 375 and 354 RT primers, respectively, will be pooled; while Pool C will contain 274 RT primers (*see* Note 24). The desired endogenous controls including 18S, RNU43, RNU38B and U6 as well as exogenous controls for the three *C. elegans* microRNAs can be added to the pools according to the microRNA template (Fig. 2).
3. The RT Primer Pools need to be prepared at a final volume of 1 mL regardless of the primer number.
4. Therefore, to reduce the volume from approximately 4 mL, divide the RT Primer Pools solution between several RNase/DNase free 1.5 mL tubes.

²²Generally, we plate several 384-well plates at a time using a liquid handling robotic system. The plated primers are good for 3–4 months, provided they are stored at -20°C .

²³If the required amount of diluted primer exceeds the maximum volume per well of the V-bottom plate, multiple plates can be made.

²⁴Alternatively, RT primer pools A and B are available for purchase through the manufacturer (Megaplex[™] RT primer Human Pools A v2.1 and B v3.0).

5. Using a speed vac, reduce the volume by approximately 75 % of the original to achieve a final volume of 1 mL for the combined RT Primer Pool solution.
6. If the resultant solution is less than 1 mL, adjust volume to achieve 1 mL with molecular grade water.
7. The RT Primer Pools are to be stored at -20°C and are good for 6–12 months.
8. In a 200 μL PCR tube, add 100 ng RNA (*see* Note 25) in a final volume of 12.75 μL then add 2.25 μL of DNase master mix (*see* Note 26).
9. Vortex then centrifuge tube for 30s in a microcentrifuge. Incubate the reaction in a thermal cycler for 10 min at 37°C followed by an incubation at 90°C for 5 min.
10. To the denatured RNA (**Step 9**), add 10 μL of the appropriate RT Primer Pool and 25 μL of Reverse Transcription Master Mix (for details *see* Subheading 2.5, **Step 4**). Final volume is 50 μL .
11. Mix and centrifuge tube for 30 s in a microcentrifuge.
12. Transfer the RT reaction to a thermal cycler and perform the following incubations. **Step 1:** 16°C for 2 min; **Step 2:** 42°C for 1 min; **Step 3:** 50°C for 1 s; Repeat **Steps 1–3** for 40 cycles followed by 5 min incubation at 85°C and a 4°C hold.
13. The resultant cDNA can be pre-amplified immediately or can be stored at -20°C .

3.10 Optional: Pre-Amplification Protocol

1. If RNA samples used for cDNA generation are of limited quantity or low concentration (between 40 and 100 ng), it is advantageous to perform a pre-amplification step prior to qRT-PCR [16].
2. Pre-Amplification Primer Mix that does not contain quencher or probe corresponding to microRNA Assay Pools A and B are commercially available through Applied Biosystems. Pre-Amplification Primer Pool C must be made using the TaqMan[®] PCR primers (*see* Notes 27 and 28).
3. To pre-amplify the cDNA for microRNA assays corresponding to Human Pools A or B, transfer 5 μL of cDNA (Subheading 3.9) to a new a PCR tube and add 2.5 μL of Megaplex[™] PreAmp Primers for either Human Pools A v2.1 or B v3.0, 12.5 μL of TaqMan[®] PreAmp Master Mix and 5 μL molecular grade water. Final volume is 25 μL .
4. To pre-amplify Pool C, the reaction consists of 5 μL of cDNA (Subheading 3.9), 5 μL of Pool C TaqMan[®] PCR primers (*see* Note 27), 12.5 μL of TaqMan[®] PreAmp Master Mix and 2.5 μL of molecular grade water. Final volume is 25 μL .
5. Mix and pulse spin down the reactions.

²⁵If using 40–100 ng of RNA then pre-amplification of the cDNA should be performed prior to qRT-PCR analysis. Ensure that the proper endogenous controls are included in the pre-amplification primer mixes.

²⁶The DNase Master Mix contains 0.9 μL of DNase I, 0.15 μL of RNase inhibitor and 1.2 μL of 25 mM MgCl_2 .

²⁷The Pool C Pre-Amp Primer Mix will be generated using the TaqMan[®] PCR primers for this Pool. Combine 10 μL of the supplied TaqMan[®] PCR primers in a sterile RNase/DNase free basin. Ensure the proper endogenous controls are included. Similar to the RT primer pools, the final volume needs to be 1 mL regardless of the number of primers. If necessary, reduce the volume using a speed vac to achieve a final volume of 1 mL. If the resultant solution is less than 1 mL, adjust volume to achieve 1 mL with molecular grade water.

²⁸According to the manufacturer, the presence of the TaqMan[®] probes in the Pool C Pre-Amp Primer Mix will not interfere with the pre-amplification step.

6. Place the reactions in a thermocycler and perform the following protocol: **Step 1:** 95 °C for 10 min; **Step 2:** 55 °C for 2 min; **Step 3:** 72 °C for 2 min; **Step 4:** 95 °C for 15 s; **Step 5:** 60 °C for 4 min; Repeat **Steps 4–5** for 12 cycles followed by a 4 °C hold.
7. Dilute the pre-amplification product 1:50 with molecular grade water and store at –20 °C until needed or proceed immediately to qRT-PCR (Subheading 3.11).

3.11 Quantitative Real-Time PCR for microRNAs

1. To profile each sample in duplicate, two 384-well plates with each Primer Pool generated in (Subheading 3.8) will be used.
2. The cDNA from Subheading 3.9 will be diluted 1:50 prior to use, while the pre-amplified cDNA (Subheading 3.10) is already diluted and will be used directly.
3. Prepare the RT-PCR Master Mix in a sterile basin for two plates by mixing 2.5 mL of TaqMan® Universal Master Mix, No AmpErase® UNG; 1.5 mL of molecular grade water and 1 mL of diluted cDNA. *Important:* Ensure that the cDNA Pool corresponds to the Primer Pool designated on the 384-well plate.
4. Using a multi-channel, repeating pipette, add 5 µL of the RT-PCR Master Mix (**Step 3**) to each well of the 384-well plates.
5. Seal plates using optical adhesive film and pulse spin to bring down any residual RT-PCR Master Mix. Store the duplicate plate in the dark at 4 °C while the first plate is running.
6. Run the samples on 7900HT Fast Real-Time PCR System using Standard mode. Cycle details are as follows: **Step 1:** 95 °C for 10 min; **Step 2:** 95 °C for 15 s; **Step 3:** 60 °C for 1 min; Repeat **Steps 2–3** for 40 cycles.
7. The cycle threshold (C_T) values are exported from SDS 2.2.3 software and analyzed in Microsoft Excel. C_T analysis settings in SDS are set manually with a threshold of 0.2 and baseline is set between cycles 3 and 15.
8. Duplicate C_T values are imported into a single spreadsheet and compared side by side. A difference of greater than 5 between duplicate C_T values is flagged and should be repeated.
9. Any C_T values ≥ 35 are excluded from the final analysis and considered undetectable.
10. Average C_T values for all microRNAs and control RNAs are determined for each sample.
11. The control microRNA(s) with the least coefficient of variation across the entire sample population are identified. An average C_T value for these microRNAs is calculated from each sample and designated as housekeeping microRNAs (HK-microRNA) used for normalization.
12. To normalize the data, subtract the HK-microRNAs (**Step 11**) from the average experimental C_T values for each sample (**Step 10**). The normalized data is the ΔC_T .
13. Calculate relative copy number (RCN) of the microRNA using the following equation: $RCN = 2^{(-\Delta C_T)}$.

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Figure 1.

Flow Cytometric Analysis of Polystyrene Bead Standards. Shown is a representative dot plot (FSC vs SSC) for extracellular vesicles and 2 μm the polystyrene bead standards using the BD LSRII flow cytometry. To quantitate the concentration of extracellular vesicles, we collect 1,000 events in the 2 μm region. Gate 5 labeled extracellular vesicles is indicated and drawn based on the polystyrene bead standards (0.07 - 1 μm) as indicated by the boxed regions. Using the equation found in Subheading 3.2 and Note 12, the concentration can be determined

	1		2		3		4		5		6		7		8		9		10		11		12	
a	Box 1 A1	Box 2 A1	Box 1 A2	Box 2 A2	Box 1 A3	Box 2 A3	Box 1 A4	Box 2 A4	Box 1 A5	Box 2 A5	Box 1 A6	Box 2 A6	Box 1 A7	Box 2 A7	Box 1 A8	Box 2 A8	Box 1 A9	Box 2 A9	Box 1 A10	Box 2 A10	Box 1 A11	Box 2 A11	Box 1 A12	Box 2 A12
	Box 3 A1	Box 4 A1	Box 3 A2	Box 4 A2	Box 3 A3	Box 4 A3	Box 3 A4	Box 4 A4	Box 3 A5	Box 4 A5	Box 3 A6	Box 4 A6	Box 3 A7	Box 4 A7	Box 3 A8	Box 4 A8	Box 3 A9	Box 4 A9	Box 3 A10	Box 4 A10	Box 3 A11	Box 4 A11	Box 3 A12	Box 4 A12
b	Box 1 B1	Box 2 B1	Box 1 B2	Box 2 B2	Box 1 B3	Box 2 B3	Box 1 B4	Box 2 B4	Box 1 B5	Box 2 B5	Box 1 B6	Box 2 B6	Box 1 B7	Box 2 B7	Box 1 B8	Box 2 B8	Box 1 B9	Box 2 B9	Box 1 B10	Box 2 B10	Box 1 B11	Box 2 B11	Box 1 B12	Box 2 B12
	Box 3 B1	Box 4 B1	Box 3 B2	Box 4 B2	Box 3 B3	Box 4 B3	Box 3 B4	Box 4 B4	Box 3 B5	Box 4 B5	Box 3 B6	Box 4 B6	Box 3 B7	Box 4 B7	Box 3 B8	Box 4 B8	Box 3 B9	Box 4 B9	Box 3 B10	Box 4 B10	Box 3 B11	Box 4 B11	Box 3 B12	Box 4 B12
c	Box 1 C1	Box 2 C1	Box 1 C2	Box 2 C2	Box 1 C3	Box 2 C3	Box 1 C4	Box 2 C4	Box 1 C5	Box 2 C5	Box 1 C6	Box 2 C6	Box 1 C7	Box 2 C7	Box 1 C8	Box 2 C8	Box 1 C9	Box 2 C9	Box 1 C10	Box 2 C10	Box 1 C11	Box 2 C11	Box 1 C12	Box 2 C12
	Box 3 C1	Box 4 C1	Box 3 C2	Box 4 C2	Box 3 C3	Box 4 C3	Box 3 C4	Box 4 C4	Box 3 C5	Box 4 C5	Box 3 C6	Box 4 C6	Box 3 C7	Box 4 C7	Box 3 C8	Box 4 C8	Box 3 C9	Box 4 C9	Box 3 C10	Box 4 C10	Box 3 C11	Box 4 C11	Box 3 C12	Box 4 C12
d	Box 1 D1	Box 2 D1	Box 1 D2	Box 2 D2	Box 1 D3	Box 2 D3	Box 1 D4	Box 2 D4	Box 1 D5	Box 2 D5	Box 1 D6	Box 2 D6	Box 1 D7	Box 2 D7	Box 1 D8	Box 2 D8	Box 1 D9	Box 2 D9	Box 1 D10	Box 2 D10	Box 1 D11	Box 2 D11	Box 1 D12	Box 2 D12
	Box 3 D1	Box 4 D1	Box 3 D2	Box 4 D2	Box 3 D3	Box 4 D3	Box 3 D4	Box 4 D4	Box 3 D5	Box 4 D5	Box 3 D6	Box 4 D6	Box 3 D7	Box 4 D7	Box 3 D8	Box 4 D8	Box 3 D9	Box 4 D9	Box 3 D10	Box 4 D10	Box 3 D11	Box 4 D11	Box 3 D12	Box 4 D12
e	Box 1 E1	Box 2 E1	Box 1 E2	Box 2 E2	Box 1 E3	Box 2 E3	Box 1 E4	Box 2 E4	Box 1 E5	Box 2 E5	Box 1 E6	Box 2 E6	Box 1 E7	Box 2 E7	Box 1 E8	Box 2 E8	Box 1 E9	Box 2 E9	Box 1 E10	Box 2 E10	Box 1 E11	Box 2 E11	Box 1 E12	Box 2 E12
	Box 3 E1	Box 4 E1	Box 3 E2	Box 4 E2	Box 3 E3	Box 4 E3	Box 3 E4	Box 4 E4	Box 3 E5	Box 4 E5	Box 3 E6	Box 4 E6	Box 3 E7	Box 4 E7	Box 3 E8	Box 4 E8	Box 3 E9	Box 4 E9	Box 3 E10	Box 4 E10	Box 3 E11	Box 4 E11	Box 3 E12	Box 4 E12
f	Box 1 F1	Box 2 F1	Box 1 F2	Box 2 F2	Box 1 F3	Box 2 F3	Box 1 F4	Box 2 F4	Box 1 F5	Box 2 F5	Box 1 F6	Box 2 F6	Box 1 F7	Box 2 F7	Box 1 F8	Box 2 F8	Box 1 F9	Box 2 F9	Box 1 F10	Box 2 F10	Box 1 F11	Box 2 F11	Box 1 F12	Box 2 F12
	Box 3 F1	Box 4 F1	Box 3 F2	Box 4 F2	Box 3 F3	Box 4 F3	Box 3 F4	Box 4 F4	Box 3 F5	Box 4 F5	Box 3 F6	Box 4 F6	Box 3 F7	Box 4 F7	Box 3 F8	Box 4 F8	Box 3 F9	Box 4 F9	Box 3 F10	Box 4 F10	Box 3 F11	Box 4 F11	Box 3 F12	Box 4 F12
g	Box 1 G1	Box 2 G1	Box 1 G2	Box 2 G2	Box 1 G3	Box 2 G3	Box 1 G4	Box 2 G4	Box 1 G5	Box 2 G5	Box 1 G6	Box 2 G6	Box 1 G7	Box 2 G7	Box 1 G8	Box 2 G8	Box 1 G9	Box 2 G9	Box 1 G10	Box 2 G10	Box 1 G11	Box 2 G11	Box 1 G12	Box 2 G12
	Box 3 G1	Box 4 G1	Box 3 G2	Box 4 G2	Box 3 G3	Box 4 G3	Box 3 G4	Box 4 G4	Box 3 G5	Box 4 G5	Box 3 G6	Box 4 G6	Box 3 G7	Box 4 G7	Box 3 G8	Box 4 G8	Box 3 G9	Box 4 G9	Box 3 G10	Box 4 G10	Box 3 G11	Box 4 G11	Box 3 G12	Box 4 G12
h	Box 1 H1	Box 2 H1	Box 1 H2	Box 2 H2	Box 1 H3	Box 2 H3	Box 1 H4	Box 2 H4	Box 1 H5	Box 2 H5	Box 1 H6	Box 2 H6	Box 1 H7	Box 2 H7	Box 1 H8	Box 2 H8	Box 1 H9	Box 2 H9	Box 1 H10	Box 2 H10	Box 1 H11	Box 2 H11	Box 1 H12	Box 2 H12
	Box 3 H1	Box 4 H1	Box 3 H2	Box 4 H2	Box 3 H3	Box 4 H3	Box 3 H4	Box 4 H4	Box 3 H5	Box 4 H5	Box 3 H6	Box 4 H6	Box 3 H7	Box 4 H7	Box 3 H8	Box 4 H8	Box 3 H9	Box 4 H9	Box 3 H10	Box 4 H10	Box 3 H11	Box 4 H11	Box 3 H12	Box 4 H12

Figure 2.

Template for arranging TaqMan® PCR primers in four 96-slot boxes to 384-well format. The 384-well plate is divided into 2 × 2 quadrants. The resultant 96 quadrants are labeled to represent a 96 well plate, with 12 columns and 8 Rows (A–H). The 96-slot boxes are designated as Box 1–4 in each quadrant. The corresponding content of A1 is then placed into the A1 position of the 384-well template. The template file is then created in Applied Biosystems' Sequence Detection System (SDS) software v2.2.3 for qRT-PCR