A STANDARDIZED, AUTOMATED APPROACH FOR EXOSOME ISOLATION AND CHARACTERIZATION USING BECKMAN COULTER INSTRUMENTATION

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ABSTRACT & INTRODUCTION:

Exosomes are small microvesicles, derived from the late endosome, most often described in the literature to be less than 120 nm, released by all cell types, and proven to be involved in cancer metastasis[1-3]. Exosome characterization and analysis comprise a fast, evolving research area even though their biological function has yet to be completely elucidated. Exosomes contain proteins, lipids, and microRNA capable of regulating an assortment of target genes. Recent studies have suggested that exosomes can serve as biomarkers for future clinical and diagnostic use in not only cancer but many other human diseases[4]. Exciting new findings have implicated exosomes in cardiovascular diseases[5-7], autoimmune syndromes[8], and neurodegenerative disorders such as Alzheimer's[9] and Parkinson's[10] disease, in addition to infectious diseases such as tuberculosis[11], diphtheria[12], and even HIV[13].

An improved and more efficient isolation and characterization protocol for exosomes and other EVs is critical to advancing this exciting field and experts have recently called for the establishment of standardized methods. EV isolation is particularly tedious, requiring several rounds of differential centrifugation and a density gradient centrifugation step to obtain highly pure vesicles. Downstream challenges involve a standardized method for genetic profiling of encapsulated miRNA. Here, we describe a workflow using automated Biomek methods for centrifugation layering and fractionation, total RNA extraction, and cDNA amplification and clean-up for next generation sequencing. NGS results are reported on benign and cancerous colon cell lines.

MATERIALS & METHODS:

Preparation of Exosome-Depleted Media: Ultracentrifuged media: 500 mL of standard HI-FBS was added equally to 6 Beckman Coulter Ultra-Clear 94 mL centrifuge tubes (part #: 345777) with an adapter and then placed in a Beckman Coulter Type 45 Ti rotor and spun at 120,000 x g, 18 hours, 4°C in a Beckman Coulter Optima XPN ultracentrifuge. The supernatant of each tube was recovered and aliquoted to 50 mL and stored in the -20°C freezer for future use. 50 mL of the centrifugally-depleted FBS was then added to 450 mL of both MEM and RPMI 1640 media. The media was finally supplemented with 10 mM HEPES and 100 U/mL Penicillin-Streptavidin.

Cell Culture: Frozen stocks of HCT 116 (Normal Colon) (ATCC CCL-247) and CCD 841 CoN (Colorectal Carcinoma) (ATCC CRL-1790) lines were thawed and suspended in the separate buffer types and initially added to 6 well culture plates (Becton Dickinson). Cells were expanded as they reached confluency and added to T-175 flasks (Greiner). Briefly, both cell lines were trypsinized, resuspended in appropriate buffer, and centrifuged at 750 x g, 10 min, 20°C in a Beckman Coulter Allegra X-15 R in an SX4750A rotor. Cells were resuspended again in the appropriate buffer and 1 mL was added to vials and placed in the Vi-Cell for analysis. 1 mL of the suspended cells was then added directly to the Vi-Cell and quantified for yield and viability.

Differential Centrifugation for Exosome Isolation: Following cell pelleting in the previous step, the cell culture media was filtered through a 0.45 µm filter and centrifuged at 2000 x g for 20 minutes at 4°C. The supernatant was then centrifuged at 10,000 x g for 30 minutes in the Optima XPN ultracentrifuge equipped with a SW 32 Ti rotor to remove cell debris. Again, the supernatant was recovered, filtered through a 0.22 µm membrane, and spun at 100,000 x g

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for 90 minutes with an SW 41 Ti rotor in an Optima XPN. This time, the supernatant was aspirated and the pellet was recovered by resuspending in phosphate buffered saline (PBS). The resuspended sample was labeled as 'crude' and is stable at -20°C for an extended period of time.

Density Gradient Centrifugation for Further Refinement: To further purify the sample, Beckman Coulter's Biomek 4000 Laboratory Automation Workstation was used to provide a quick, consistent, and reproducible method for layering a centrifugation density gradient with the volumes and density shown in Figure 2. The resuspended crude exosome sample was then layered on top of the gradient and centrifuged at 100,000 x g at 4°C for 18 hours with an SW 41 Ti rotor and Optima XPN. The Biomek 4000 was employed again to fractionate the gradient after the centrifugation step. 1 mL fractions were collected from the top using liquid level tracking for a total of 13 fractions which were then pelleted using a TLA 120.2 rotor in an Optima Max-XP bench-top centrifuge. The resulting pellet was again resuspended in PBS, analyzed for size, and based on expected density and size of recovered exosomes, fractions 7-9 were combined, pelleted once more using the TLA 120.2 rotor, and finally resuspended in a small volume of PBS.

Particle Characterization: The highly purified fractions were again analyzed by the Beckman Coulter DelsaMax for size. The translational diffusion coefficients D and hydrodynamic diameters D_h were determined from an autocorrelation analysis of the scattered light at 514.5 nm on a Beckman Coulter DelsaMax Pro. Twenty DLS acquisitions of 5 seconds each were ran with no auto-attenuation with to and peak radius cut-offs between 0.5 nm and 150 nm. Data was exported from the DelsaMax software package and put into Origin for plotted.

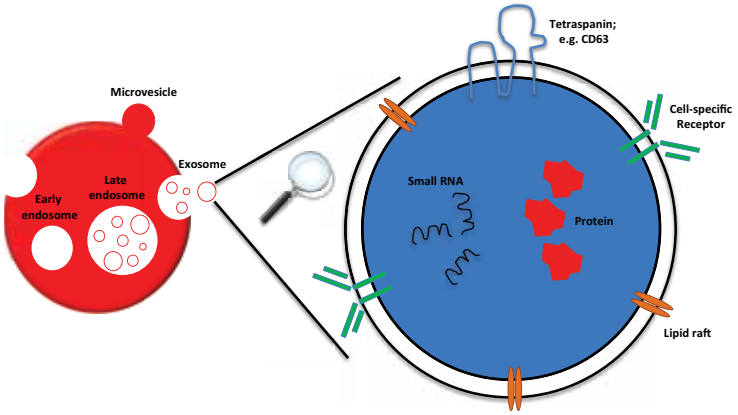
RNA Extraction: Qiagen's miRNeasy kits were used to extract total RNA from highly purified exosomes of both cell lines and both the highly purified and crude exosomes. The extracted RNA was analyzed on a Thermo Scientific NanoDrop 8000 for concentration and an Agilent BioAnalyzer Pico Chip for size determination.

NGS Library Preparation, Sequencing, and Analysis: Exosome RNA from each pair of fractions was quantified using Quant-iT RiboGreen (Life Technologies) with a SpectraMax i3 plate fluorometer (Molecular Devices). Approximately 100ng of each exosome RNA samples were converted into Illumina compatible small RNA libraries using the NEBNext Small RNA Library Preparation Kit for Illumina (New England Biolabs) automated on the Biomek 4000 automated liquid handler (Beckman Coulter, Inc.). Size selection was performed using SPRI-based size selection. Following library construction, the libraries were assayed on the BioAnalyzer 2100 (Agilent) using a DNA High Sensitivity Chip. The libraries were then sequenced using an Illumina MiSeq with a 50 cycle single read sequencing run. Following the sequencing run, the data was analyzed using the Illumina BaseSpace Small RNA application.

RESULTS & DISCUSSION:

Exosomes are the smallest subset of extracellular vesicles, comprised of tetraspanins, cell-specific receptors, lipid rafts, proteins, and small RNA (Fig. 1). Exosomes are involved in cell-to-cell communication and the message encapsulated inside their lipid-derived shell is critical to homeostasis. Recently, exosomes have been researched for their therapeutic capabilities as a nano-delivery device to diseased cells.

Figure 1. Schematic of an exosome budding from a cell and magnified to show major components.



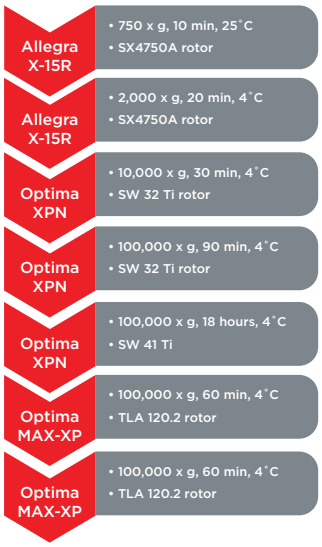
Exosomes are derived from many sources including nearly all bodily fluids, cell types, and species; however, cell culture remains a popular approach for the study of extracellular vesicles. Beckman Coulter's Vi-Cell is an automated viability counter that was used to assess the number and viability of both the HCT 116 and CCD 841 CoN cells. The cells were highly viable at 97.3% and 98.4% for HCT 116 and CCD 841 CoN cell, respectively (Table 1) at a density of 1.52 x 10^8 and 0.82 x 10^8, respectively.

Table 1. Cell Number and Viability of Two Colon Cell Lines

CELL TYPE	DESCRIPTION	CELL COUNT	CELL VIABILITY
HCT 116	Colorectal Carcinoma	1.52 x 10^8	97.3%
CCD 841 CoN	Normal Colon	0.82 x 10^8	98.4%

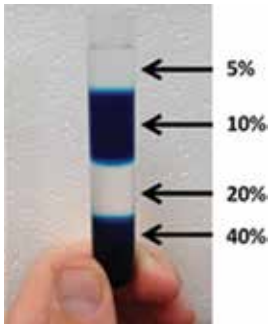
Several differential centrifugation steps and a density gradient are required to separate whole cells, cell debris, large aggregates, and soluble proteins from the vesicles of interest. These steps are detailed in Figure 2. The experimental method follows two paths; one in which the exosome isolation is terminated following the fourth centrifugal spin (100,000 x g pellet) and the other that goes through the density gradient separation and 2 additional pelleting steps. The starting material for both protocols was equal as initial cell input was aliquoted accordingly. The Biomek 4000 Workstation was utilized in the density gradient workflow to both layer the separation media and fractionate the samples to reduce the variability between run and decrease the experimental hands-on time.

Figure 2. Typical centrifugation workflow and iodixanol gradient setup for stringent purification of exosomes from cell culture. Gradients were layered and fractionated using a Biomek 4000.



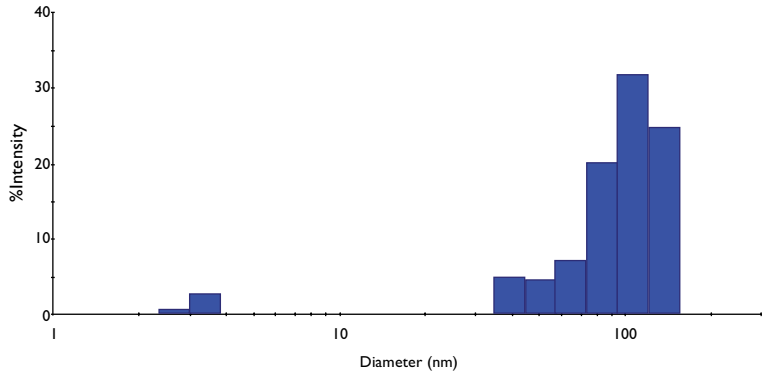
Centrifugation Workflow for Exosome Isolation

GRADIENT LAYER	ESTIMATED DENSITY (g/ml)	VOLUME (ml)	% IODIXANOL (0.25 M S crose;ph 7.5)
1	1.223	3	40
2	1.127	3	20
3	1.079	3	10
4	1.054	2	5



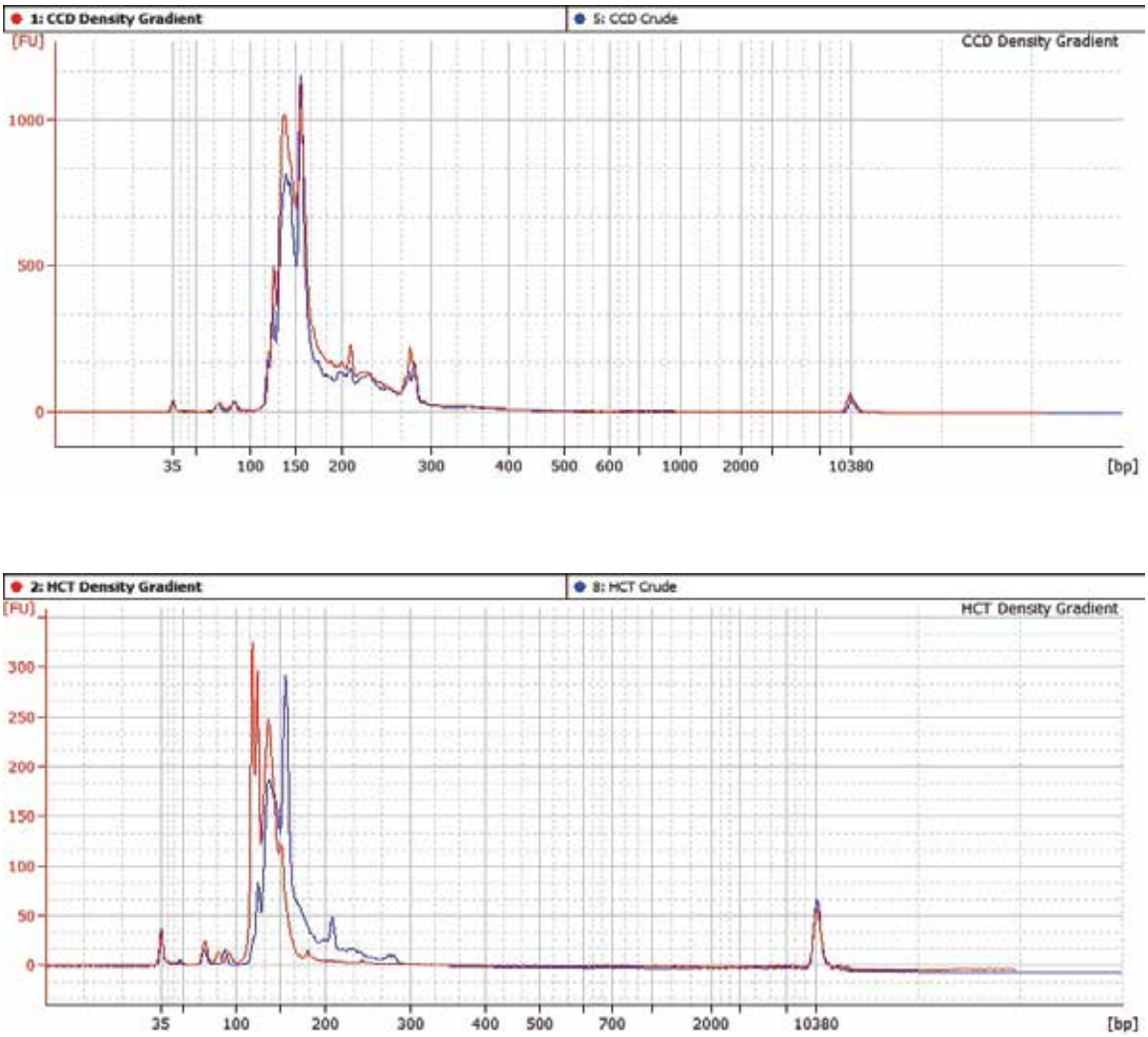
Following isolation, the exosomes were sized by dynamic light scattering using Beckman Coulter's DelsaMax Core. In all cases, the purified exosomes were of the appropriate size between 30 and 150 nm but with some residual proteins or other particles around 5 nm. Data for HCT 116 crude exosomes are shown (Fig. 3).

Figure 3. Representative plot of DLS data acquired from purified exosomes.



Total RNA was then extracted, quantified, and sized. Exosomes contain a wide variety of RNA of different sizes which is evident in the BioAnalyzer tracers (Fig. 4). The RNA had broad peaks centered between 20 and 30 nucleotides suggesting a large miRNA population but also contained peaks representative or other RNA species such as mRNA, ribosomal RNA and precursor RNA as probed by the Pico RNA chip. Surprisingly, the RNA obtained from the crude exosomes and the density gradient purified exosomes were very similar in size, but the concentration of the RNA was significantly higher in that from the density gradient.

Figure 4. Electrophoretic BioAnalyzer trace of exosomal total RNA derived from CCD 841 CoN (top) and HCT 116 (bottom) cells isolated with (red) or without (blue) a density gradient.

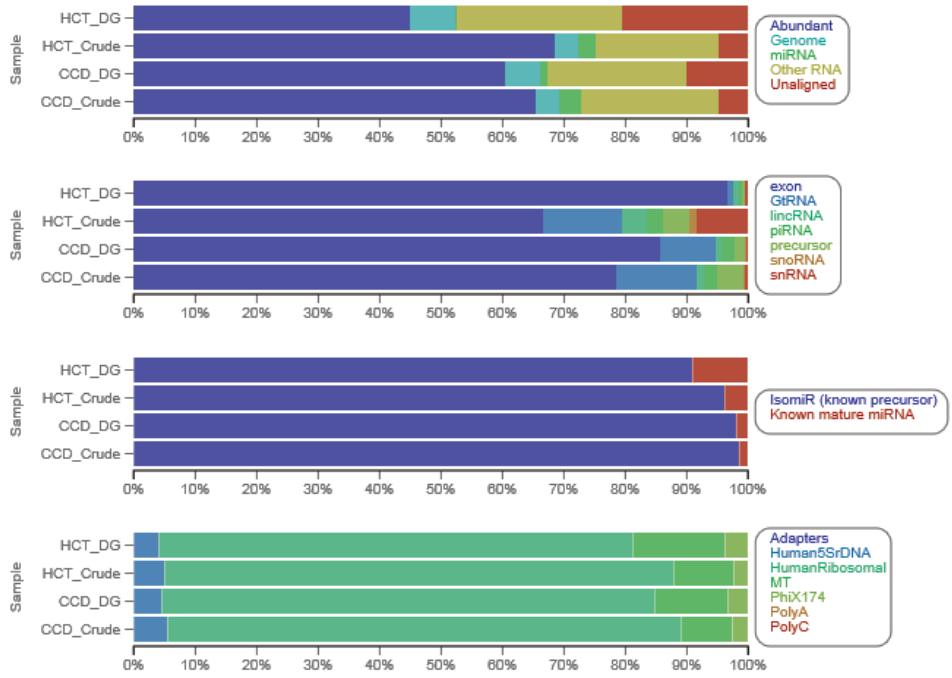


RNA isolated from each exosomal samples was prepared into Illumina compatible Small RNA sequencing libraries using the NEBNext Small RNA Library Preparation Kit for Illumina on the Biomek 4000 Genomic Workstation. Libraries were quantified using the Kapa Biosystems Illumina Library Quantification kit. RNA from crude (ultracentrifugation only) and density gradient preps from HCT and CCD cell lines were prepared into small RNA libraries for next generation sequencing (NGS) and sequenced on an Illumina MiSeq sequencer using a 50 cycle v2 sequencing kit.

Following FASTQ generation and read trimming, sequencing analysis was performed on BaseSpace using the Small RNA Application, which employs BowTie alignment to the hg19 human reference genome and miRDeep and DESeq2 for differential expression. CCD 841 CoN and HCT 116 density gradient exosomal RNA generated 678,231 and 600,307 PassFilter read counts, respectively, whereas CCD 841 CoN and HCT 116 crude exosomal RNA created 660,025 and 617,001 read counts, respectively. The high number of reads and low variation between data sets suggests that the isolated RNA is robust and at high enough yield for sequencing.

There was significant variation in RNA type between cell lines and preparation methods (Fig. 5). In the top plot, it is evident that the total relative abundance of miRNA is the lowest in the HCT 116 density gradient preparation method, but that mature miRNA is actually the greatest in this prep (third plot). The most variation between preparation methods was evident in the HCT 116 cells. In terms of small RNA, significantly more exons were present in the density gradient prep but this translated to additional GtRNA, long, non-coding RNA, piRNA, precursor RNA, snoRNA, and snRNA for the crude prep. Of the abundant RNA, human ribosomal RNA is the most prevalent in all cell lines and prep methods.

Figure 5. Relative abundance chart of RNA type following FASTQ generation and read-trimming.



Expression heat maps of the sequenced precursor and mature miRNA were also plotted and illustrated (Fig. 6). The plot represents differential expression between preparation methods within a cell line. Through further analysis, it was evident that many miRNA families also had significant differential expression between cancerous and normal colon cell lines (Fig. 7). Fifteen gene families were determined to be significantly differentially-expressed. Of note, mir-1246, mir-182, and mir-183 were all significantly up-regulated (>3.75 fold change) in the colon cancer cell line, CCD 841 CoN. In fact, these three gene families have previously been identified to be upregulated in colon cancer[14-16] aligning well with our results.

Figure 6. Differential expression heat-map of preparation method within a single cell line. Both precursor miRNAs and mature miRNAs are plotted.

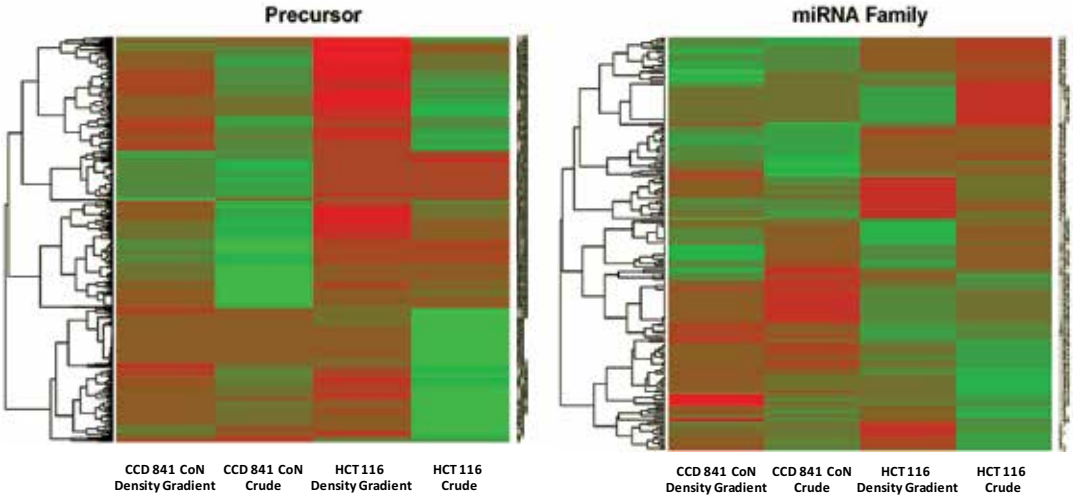
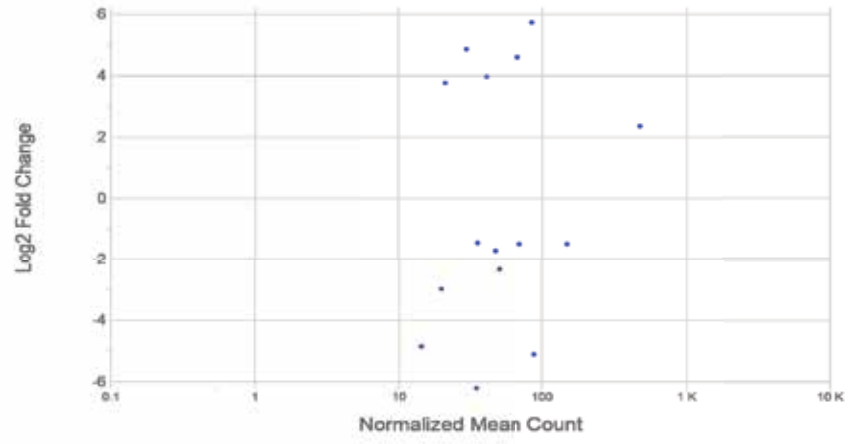


Figure 7. Differential expression of miRNA read counts within a sequencing library between HCT116 and CCD 841 CoN cells.

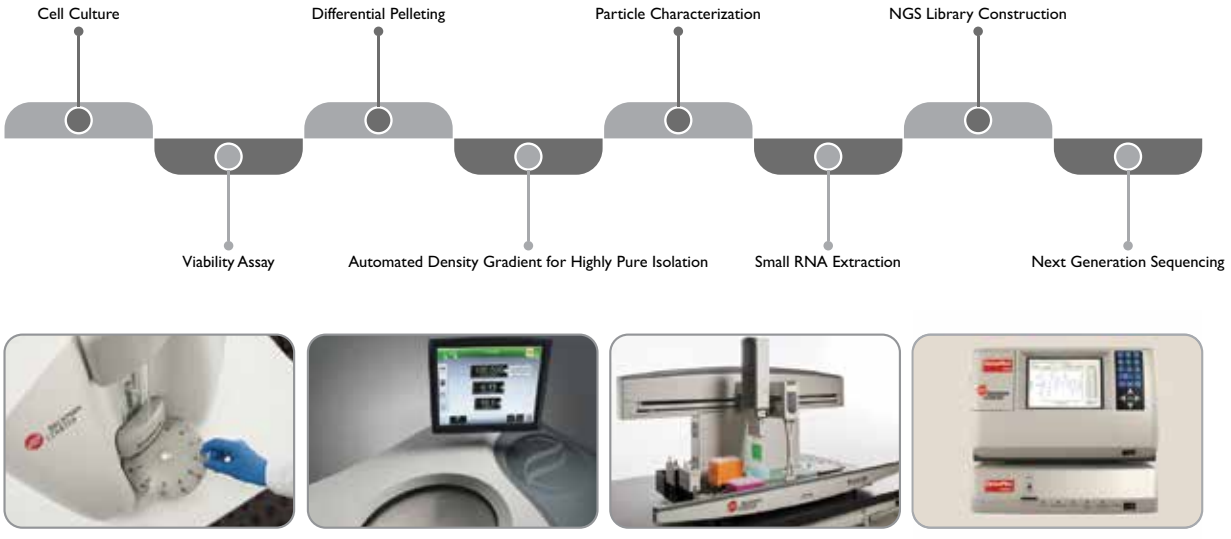


miRNA FAMILY	MEAN COUNT	LOG_2 (FOLD CHANGE)	STD. ERR. LOG_2 (FOLD CHANGE)	q VALUE	REGULATION IN COLON CANCER	SOURCE
mir-1246	41	3.98	0.733	6.07E-07	Up	Ogata-Kawata, Hiroko et al., 2014
mir-182	29.7	4.87	1.04	0.0000184	Up	Perilli, Lisa et al., 2014
mir-183	66.7	4.61	1.09	0.000114	Up	Zhou et. al., 2014

CONCLUSIONS:

Standardization of isolation and characterization methods is critical to the advancement of this exciting, emerging field. Density gradient ultracentrifugation is frequently the preferred choice for exosome isolation, generating highly pure sample preparations; however, the workflow often lacks reproducibility among laboratories and users. Next generation small RNA sequencing is one of many downstream assays for exosome characterization and biomarker identification, but again the protocols employed often vary drastically. Here we presented a solution using the breadth of Beckman Coulter instrumentation to increase throughput, walk-away time, reproducibility, and accuracy of results (Fig. 8). The results demonstrated a workflow capable of producing high impact next generation sequencing data with only a single MiSeq sequencing run which can be used to identify potential biomarkers and measure differential expression against sample type.

Figure 8. Beckman Coulter's standardized exosome workflow.



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