Exosome Isolation and Characterisation: A Standardised, Automated Approach with Ultracentrifugation

Dr Chad Schwartz and Zach Smith, Beckman Coulter Life Sciences, Indianapolis, IN 46268.

Centrifugation using automated liquid handling now offers an improved, more efficient and standardised exosome isolation protocol, as demonstrated by work reported by Chad Schwartz PhD and Zach Smith M.S.

Exosomes are small microvesicles, derived from late endosome, released by all cell type and are already implicated in cancer metastasis [1-3]. They are found in nearly all bodily fluids, cell types and species, and are involved in cell-to-cell communication, with the information encapsulated within their lipid-derived shell critical to homeostasis.

Recent studies suggest they serve as biomarkers not only for clinical and diagnostic use in cancer but in many other diseases [4]. 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 tuberculosis [11], diphtheria [12] and human immunodeficiency virus (HIV) [13].

Exosome characterisation and analysis is therefore a fast-evolving research area. However, an improved and more efficient isolation and characterisation protocol for exosomes and other extracellular vesicles (EVs) is critical to advancing this field, and experts have recently called for the establishment of standardised methods.

Integrated Workflow *

Density gradient, combined with differential centrifugation, is an important step in the enrichment and isolation of exosomes from cell culture media. Challenges involve several rounds of differential centrifugation and development of a standardised method for genetic profiling of exosome encapsulated miRNA.

To maximise the benefits of this technique, the authors explained the advantages of a workflow protocol incorporating automated Biomek (Beckman Coulter) methods for centrifugal layering and fractionation, total RNA extraction and cDNA amplification and clean-up for next-generation sequencing, with results reported on benign and malignant colonic cell lines.

The seven-step methodology included*:

- preparation of ultracentrifuged exosome-depleted media
- cell culture
- differential centrifugation for exosomes
- density gradient centrifugation
- particle characterisation
- RNA extraction
- NGS library preparation.

Not all the steps can be discussed in this article. Full details of the materials and methods used may be found at http://info.beckmancoulter.com/ExosomeIsolation [14].

Centrifugation and Isolation

For the preparation of exosome-depleted media, 500 mL of standard HI-FBS was added equally to six 94 mL centrifuge tubes with an adapter and then spun in an ultracentrifuge at 120,000 x g, 18 hours, 4˚C. The supernatant of each tube was recovered and aliquoted to 50 mL and stored at -20˚C 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.

While cell culture remains a popular approach to study EVs, the researchers used an automated viability counter (Beckman Coulter's Vi-Cell) to assess the number and viability of HCT 116 (normal colon) and CCD 841 CoN (colorectal carcinoma) cells, with levels of 97.3% and 98.4%, and densities of 1.52 x 10^8 and 0.82 x 10^8, respectively.

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. *Figure 1*. The experimental workflow outlined by the authors follows two paths, one in which exosome isolation is terminated following the fourth centrifugation (100,000 xg pellet) and the other that includes density gradient separation and two additional pelleting steps; however, the starting material for both protocols was the same.

The Biomek 4000 workstation (Beckman Coulter) was utilised in the density gradient workflow to layer the separation medium and fractionate the samples to reduce variability

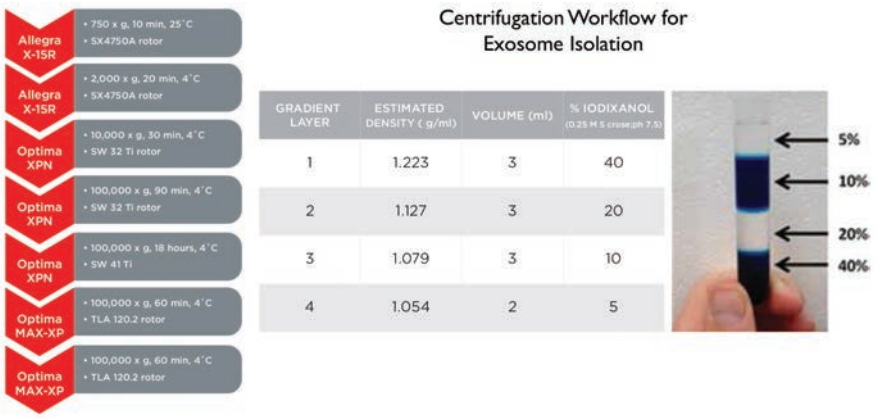

Figure 1. Typical centrifugation workflow and iodixanol gradient setup for stringent purification of exosomes from cell culture.

between runs and decrease hands-on time. Following isolation, the exosomes were sized by dynamic light scattering, and in all cases the purified exosomes were between 30 nm and 150 nm in size, but with some residual proteins or other particles of around 5 nm. Data for HCT 116 crude exosomes are shown in *Figure 2*.

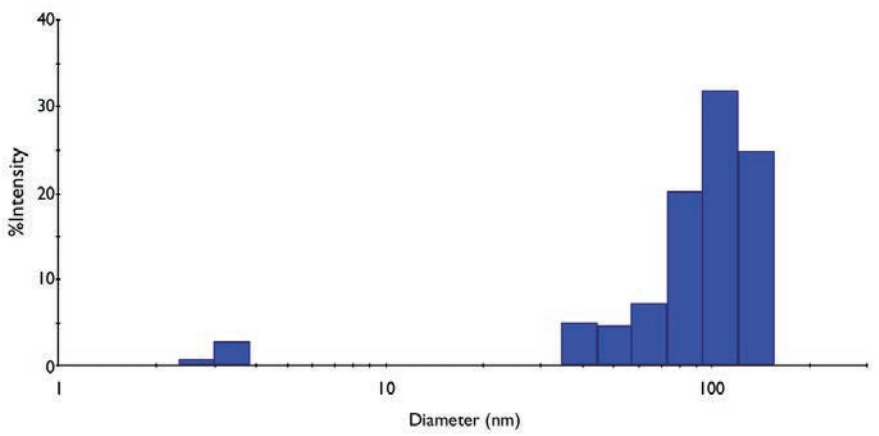

Figure 2. Representative plot of DLS data acquired from purified exosomes.

Delivers Higher Concentration

Exosomes contain RNA of different sizes, producing BioAnalyzer broad peaks of between 20 and 30 nucleotides, suggesting a large microRNA (miRNA) population, with other peaks representative of messenger RNA (mRNA), ribosomal RNA (rRNA) and precursor RNA. While the RNA obtained from the crude exosomes and the density gradient-purified exosomes were similar in size, it was noted that the concentration of the RNA obtained by density gradient ultracentrifugation was significantly higher.

The RNA isolated from each exosomal sample was prepared into small RNA sequencing libraries, using a preparation kit on the Biomek workstation. The RNA from crude (ultracentrifugation only) and density gradient preparations from the HCT and CCD cell lines were prepared into small RNA libraries for NGS using a 50-cycle sequencing kit. The CCD 841 CoN and HCT 116 density gradient exosomal RNA generated 678,231 and 600,307 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 suggested that the isolated RNA is robust and of high enough yield for sequencing.

Variation and Upregulation

The authors' results showed significant variation in RNA type between cell lines and preparation methods (*Figure 3*). 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 preparation (third plot). The greatest variation between preparation methods was evident with the HCT 116 cells. In terms of small RNA, significantly more exons were present in the density gradient preparation, but this translated into additional GtRNA, long non-coding RNA, piRNA, precursor RNA, snoRNA and snRNA for the crude preparation. Of the abundant RNA, human rRNA was the most prevalent in all cell lines and preparation methods.

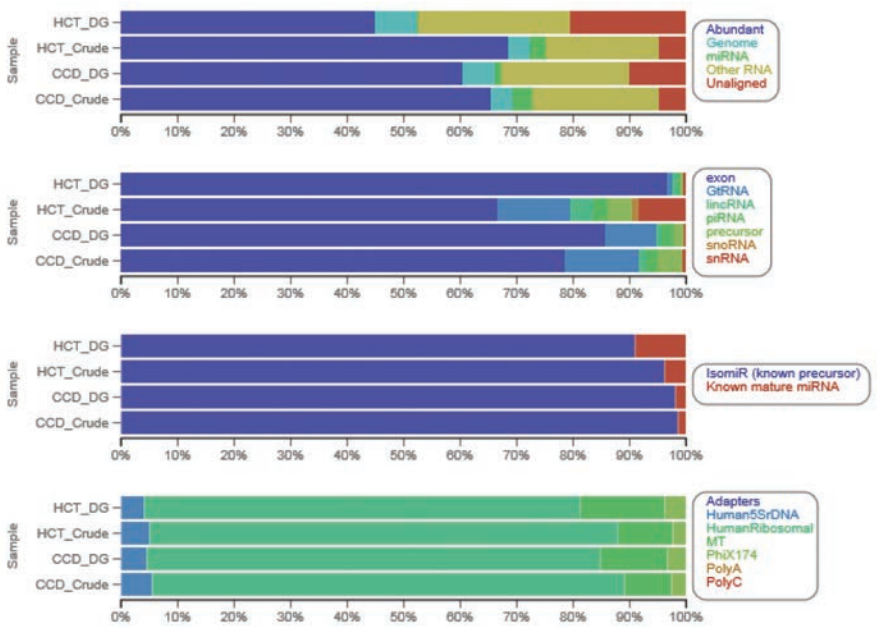

Figure 3. Relative abundance chart of RNA type following FASTQ generation and read-trimming.

Expression heat maps of the sequenced precursor and mature miRNA were also plotted to represent differential expression between preparation methods within a cell line. However, by further analysis, it was evident that many miRNA families also had significant differential expression between cancer and normal colon cell lines. 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 [13, 16, 17], aligning well with the present authors' results.

An Emerging Field

Standardisation of isolation and characterisation methods is critical to advances in this emerging field. Density gradient ultracentrifugation is frequently the preferred choice for exosome isolation, generating pure sample preparations; however, the workflow often lacks reproducibility between laboratories and users. Next-generation small RNA sequencing is one of many downstream assays for exosome characterisation and biomarker identification, but the protocols employed often vary drastically.

Here, the authors introduce a solution that uses a breadth of Beckman Coulter instrumentation to increase throughput, walkaway time, reproducibility, and accuracy of results (*Table 1*). By combining automation with the benefits of density gradient ultracentrifugation, the results demonstrate a workflow capable of producing high-impact NGS data from a single sequencing run, which can be used to identify potential biomarkers and measure differential expression against sample type.

Table 1. Standardised exosome workflow.

Cell culture
Viability assay
Differential pelleting
Automated density gradient for highly pure isolation
Particle characterisation
Small RNA extraction
NGS library construction
Next-generation sequencing

For further information on Beckman Coulter centrifugation: beckman.com/centrifugation and http://info.beckmancoulter.com/ExosomeIsolation

**Collaborator systems and processes used included: Qiagen's miRNeasy kits; Thermo Scientific NanoDrop 8000; Agilent BioAnalyzer Pico Chip/ BioAnalyzer 2100; Beckman Coulter Optima XPN ultracentrifuge with 45 Ti and SW32 Ti and SW41 Ti rotors; Allegra X-15 R with SX4750A rotor; Optima Max-XP benchtop centrifuge with TLA 120.2 rotor; Vi-Cell Viability Counter; DelsaMax Pro; Biomek 4000 Laboratory Automation Workstation; Greiner T-175 flasks; Becton Dickinson cell culture plates; Quant-iT RiboGreen (Life Technologies) with SpectraMax i3 plate fluorometer (Molecular Devices); NEBNext Small RNA Library Preparation Kit for Illumina (New England Biolabs); Ilumina MiSeq with 50 cycle single read/ Illumina BaseSpace Small RNA application.*

References

1. Vader P, Breakefield XO, Wood MJ. Extracellular vesicles: emerging targets for cancer therapy. Trends Mol Med 2014; 20 (7): 385–93.
2. El Andaloussi S, Mager I, Breakefield XO, Wood MJ. Extracellular vesicles: biology and emerging therapeutic opportunities. Nat Rev Drug Discov 2013; 12 (5): 347–57.
3. Simpson RJ, Lim JW, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential. Expert Rev Proteomics 2009; 6 (3): 267–83.
4. De Toro J, Herschlik L, Waldner C, Mongini C. Emerging roles of exosomes in normal and pathological conditions: new insights for diagnosis and therapeutic applications. Front Immunol 2015; 6: 203.
5. Amabile N, Rautou PE, Tedgui A, Boulanger CM. Microparticles: key protagonists in cardiovascular disorders. Semin Thromb Hemost 2010; 36 (8): 907–16.
6. DeJong OG, Verhaar MC, Chen Y et.al. Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes. J Extracell Vesicles 2012; 1: doi: 10.3402/jev.v1i0.18396.
7. Waldenström A, Gennebäck N, Hellman U, Ronquist G. Cardiomyocyte microvesicles contain DNA/RNA and convey biological messages to target cells. PLoS One 2012; 7 (4): e34653.
8. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. Nat Rev Immunol 2014; 14 (3): 195–208.
9. Rajendran L, Honsho M, Zahn TR et al. Alzheimer's disease beta-amyloid peptides are released in association with exosomes. Proc Natl Acad Sci USA 2006; 103 (30): 11172–7.
10. Danzer KM, Kranich LR, Ruf WP et al. Exosomal cell-to-cell transmission of alpha synuclein oligomers. Mol Neurodegener 2012; 7: 42.
11. Kruh-Garcia NA, Wolfe LM, Chaisson LH et al. Detection of Mycobacterium tuberculosis peptides in the exosomes of patients with active and latent M. tuberculosis infection using MRM-MS. PLoS One 2014; 9 (7): e103811.
12. Colino J, Snapper CM. Exosomes from bone marrow dendritic cells pulsed with diphtheria toxoid preferentially induce type 1 antigen-specific IgG responses in naïve recipients in the absence of free antigen. J Immunol 2006; 177 (6): 3757–62.
13. Gould SJ, Booth AM, Hildreth JE. The Trojan exosome hypothesis. Proc Natl Acad Sci USA 2003; 100 (19): 10592–7.
14. Ogata-Kawata H, Izumiya M, Kurioka D et al. Circulating exosomal microRNAs as biomarkers of colon cancer. PLoS One 2014; 9 (4): e92921.
15. Swartz C, Smith Z. A standardized, automated approach for exosome isolation and characterization using Beckman Coulter instrumentation (http://info.beckmancoulter.com/ExosomeIsolation).
16. Perilli L, Vicentini C, Agostini M et al. Circulating miR-182 is a biomarker of colorectal adenocarcinoma progression. Oncotarget 2014; 5 (16): 6611–9.
17. Zhou T, Zhang GJ, Zhou H, Xiao HX, Li Y. Overexpression of microRNA-183 in human colorectal cancer and its clinical significance. Eur J Gastroenterol Hepatol 2014; 26 (2): 229–33.