To the Standardization of Exosome Isolation and Characterization

Chad Schwartz, Ph.D., Zach Smith, M.S.
Beckman Coulter, Inc., Indianapolis, IN, 46268

ABSTRACT: Research involving extracellular vesicles (EVs) is rapidly expanding due to evidence suggesting a role in cancer metastasis and cell-to-cell communication, with a vast increase in the quantity of publications over the last 5 years. An improved and more efficient isolation and characterization protocol for 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.

INTRODUCTION

Exosomes are small vesicles, most often described in the literature to be less than 150 nm and have been demonstrated to be released by a wide variety of cell types, exhibiting a multitude of functions, and proven to be involved in cancer metastasis. 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 present a solution using the Biomek 4000 Genomic Workstation to increase throughput, walk-away time, reproducibility, and accuracy of results.

METHODS

CELL CULTURE

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 and offers cell-type differentiators for optimized cell counts. Researchers can analyze the number and health of their cell preparations in a quick, easy, and non-biased approach over traditional hemocytometer protocols

DIFFERENTIAL CENTRIFUGATION

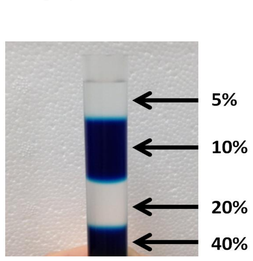
Several differential centrifugation steps are required to remove whole cells, cell debris, and large aggregates prior to density gradient isolation. These steps are detailed in the figure below.

- Allegra X-15R: • 750 x g, 10 min, 25˚C • SX4750A rotor
- Allegra X-15R: • 2,000 x g, 20 min, 4˚C • SX4750A rotor
- Optima XPN: • 10,000 x g, 30 min, 4˚C • SW 32 Ti rotor
- Optima XPN: • 100,000 x g, 90 min, 4˚C • SW 32 Ti rotor

GRADIENT LAYERING

Density gradient centrifugation generates highly pure extracellular vesicles. By utilizing different densities of gradient solutions layered in succession, exosomes and other biomolecules, such as proteins, separate based on density. To improve this tedious and often non-reproducible protocol, Biomek 4000 Genomic Workstation was employed to construct the iodixanol density gradient with high precision.

Gradient Layer	Estimated Density (g/mL)	Volume (mL)	% Iodixanol (0.25M Sucrose; pH 7.5)
1	1.223	3	40
2	1.127	3	20
3	1.079	3	10
4	1.054	2	5



DENSITY GRADIENT CENTRIFUGATION

Several centrifugal steps are required for highly pure exosome samples; Beckman Coulter, a world leader in centrifugation, offers a centrifuge and rotor for each step. After density gradient fractionation, a one-hour spin is used to re-pellet exosomes for low-volume resuspension.

- Optima XPN: • 100,000 x g, 18 hours, 4˚C • SW 41 rotor
- Optima MAX-XP: • 100,000 x g, 60 min, 4˚C • TLA 120.2 rotor

GRADIENT FRACTIONATION AND NGS LIBRARY PREPARATION

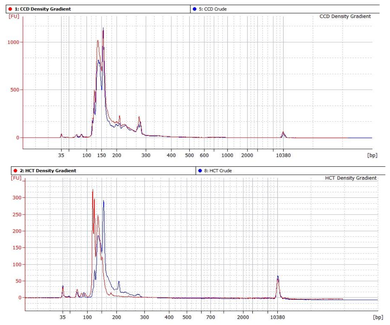
Following density gradient centrifugation, the Biomek 4000 Genomic Workstation was used to fractionate the density gradients into 1ml fractions and can be added directly into centrifuge tubes for re-pelleting. These fractions were subjected to a brief spin to pellet the exosomes, followed by RNA isolation.

RNA isolated from 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.

RESULTS & DISCUSSION

CHARACTERIZATION

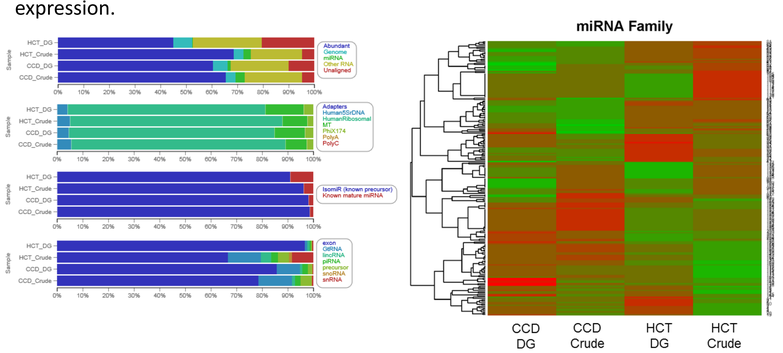
RNA from crude (ultracentrifugation only) and density gradient preps from HCT and CCD cell lines were prepared into small RNA libraries for next generation sequencing (NGS) as shown previously and sequenced on an Illumina MiSeq sequencer using a 50 cycle v2 sequencing kit.



Top: CCD NEBNext Small Libraries from Density Gradient (red) and Crude (blue) samples.
Bottom: HCT NEBNext Small Libraries from Density Gradient (red) and Crude (blue) samples.

Library	PassFilter Read Counts
CCD Density Gradient	678,231
CCD Crude	660,025
HCT Density Gradient	600,307
HCT Crude	617,001

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.



CONCLUSIONS

Hands-on time and user-to-user variation was reduced versus the manual method suggesting a robust, high-throughput protocol. The results demonstrate a workflow capable of producing high impact next generation sequencing data which can be used to identify potential biomarkers and measure differential expression against sample type.

Content Approved Code: AAG-1154PST10.15-A