APPLICATION NOTE

MicroRNA Extraction from Purified Exosomes Using Beckman's Agencourt RNAdvance Cell v2 Kits and the Biomek NX^P MC Platform

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Summary

Exosomes are ~30 nm-150 nm membrane vesicles secreted by most cell types, in vivo and vitro and these extra cellular vesicles are found in different body fluids such as blood, urine, amniotic and malignant ascites fluids. Tumor and normal cells might contain distinct subsets of microRNA (miRNA) expression profiles. Therefore, monitoring these circulating exosomal miRNAs can be used as biomarkers for early cancer screening and detection.

Exosomes can be purified either by using a traditional ultracentrifugation procedure or by using precipitation protocols. This application note describes the extraction of miRNA and RNA from precipitation purified exosomes derived from cell culture media and serum, using Beckman's Agencourt RNAdvance Cell v2 kit on a Biomek automation workstation. Automating SPRI (Solid Phase Reversible Immobilization) chemistry provides an easy, automation friendly and robust nucleic acid purification process that does not require centrifugation and vacuum filtration steps. Purified nucleic acids are easily eluted from the paramagnetic beads under aqueous conditions, which provide maximum flexibility for downstream. The RNAdvance cell Biomek method extracted 96 samples in a 96 well plate format in less than 2 hours and provides a streamlined workflow for downstream assays such as qPCR, micro-array and NGS-RNA sequencing applications.

Materials and Methods

Exosomes derived from SKOV3 or A7890 cell culture media were purified using Total Exosome Isolation Reagent (TEI) (Life Technologies, 4478360). Exosomes derived from serum were purified using ExoQuick Exosome Precipitation Solution (System Biosciences, EXOQ5A-1). 250µL of mouse serum (LAMPIRE Biological Laboratories, 7324300) was centrifuged at 3000 x g for 15 minutes to remove cells and debris, the clarified serum supernatant was transferred to a new tube and mixed with 60µL of ExosQuick solution and incubated at 4 degrees for 30 minutes, then centrifuged at 1500 x g for 30 minutes using Microfuge® 18 Microcentrifuge (Beckman Coulter, 367160). The exosome pellet was re-suspended in 25µL of 1x PBS for RNA extraction. Cells were grown to at least 10% confluency before adding exosome collection media (EMEM or RPMI 1640) containing 10% FBS. Endogenous bovine exosomes from the FBS were pelleted and removed prior to cell incubation by ultracentrifugation at 100,000 x g for 18 hours (Beckman Coulter Optima L-80 XP Preparative Ultracentrifuge, SW 32 Ti rotor). Cells were incubated for about 24-48 hours until the cell growth reached a plateau. 7.5mL of the total exosome isolation reagent was mixed thoroughly into 15mL of cultured media, the sample mixture was incubated at 4 degrees overnight and then centrifuged at 10,000 x g (Beckman Coulter, Microfuge® 18 Microcentrifuge) for 1 hours at 4 degrees. The supernatant was discarded and the pellet was re-suspended in 170µL of 1x PSBS. 1µL exosome samples were diluted 5x and lysed (RIPA Lysis Buffer, Upstate®, 20-188) for protein concentration measurement by BCA assay (Pierce® BCA Protein Assay Kit, 23225). A second aliquot was diluted 1500x in freshly filtered (via 0.1 µm Nylon syringe filter) PBS and exosome size distribution and number concentration measured by Nanoparticle Tracking Analysis (NTA, NanoSight LM10) at room temperature (data not shown). 20µL of the cell culture purified exosome (protein concentration between 3.5mg/mL-5mg/mL) or 25µL of serum purified exosome was digested with 60 µL of lysis buffer and 3µL of proteinase K for

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30 min at room temperature. The lysate was transferred on a Biomek NX^P Multi-Channel automated workstation for miRNA/RNA extraction using an RNAdvance Cell v2 kit (Beckman Coulter, A47942) following the RNAdvance Cell v2 miRNA supplemental protocol (Beckman Coulter, AAG-850SP03.15-A). RNA was eluted in 40µL of nuclease-free water in the final elution steps. 1µL of RNA was analyzed by an Agilent RNA 6000 Pico chip for concentration determination and a small RNA chip for quality determination (Agilent Technologies, 5067-1513 and 5067-1548) using the 2100 Bioanalyzer (Agilent Technologies). miRNA (let7c, miR16, miR21, miR200 and miR205) gene expression was determined by Taqman microRNA assays (Life Technologies, assay ID000379, 000391, 000397, 002623 and 000509 and 002251). 2-5µL of RNA was used for the reverse transcription reaction using the TaqMan micro RNA Reverse Transcription Kit (Life Technologies, 4366596) and 1 µL of cDNA was used per 10 µL PCR reaction in triplicate, using Taqman Universal Master Mix II (Life Technologies, 4440038). For messenger RNA gene expression, 5µL of RNA was used for cDNA synthesis using a random primer (Life Technologies, 4368814), and 1 µL of the cDNA was used per 10µL PCR reaction using prime time qPCR assays (Integrated DNA Technologies). The primer probe assay ID's used for the ACTB, B2M, GAPDH and HPRT1 were Hs.PT.39a.22214847, Hs.PT.39a.22214845, Hs.PT.39a.22214836 and Hs.PT.39a.22214821 respectively.) Details of the RT and PCR set up are described in AAG-700APP11.14-B (Reference 2). For the consumables and tools used for the Biomek NX^P Multi-Channel automated workstation, see Table 1.

Table 1: Tools and consumables needed for 96 samples.

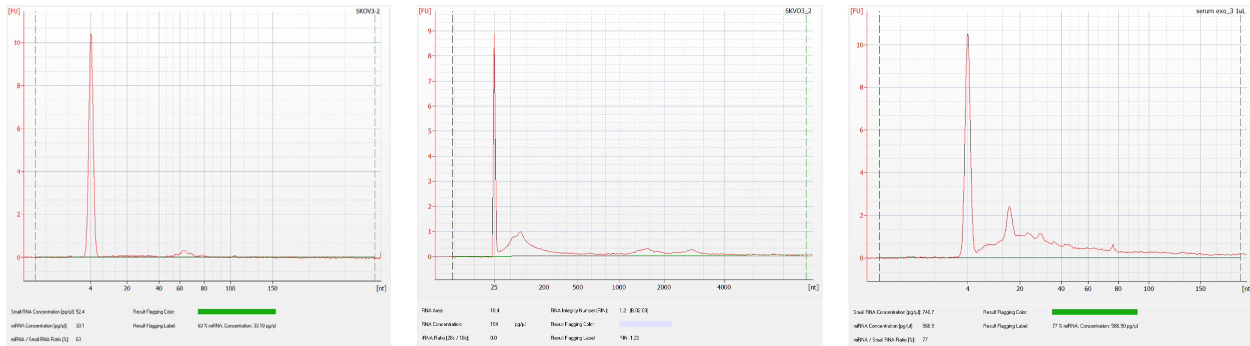
TYPE	QUANTITY	DESCRIPTION AND PART NUMBERS	VENDOR
Devices	1	Orbital Shaker, 379448	Beckman
ALPS	1	Biomek NX^P Span-8 4x3 ALP Kit, 989839	Beckman
Magnet Plate	1	Agencourt SPRIPlate 96R-Ring Super Magnet Plate, A32782	Beckman
Reservoirs	2	Pyramidal bottom 300mL Sterile Reservoir, RPI3014-ST	Phenix
Consumables	3 2 2 2 1	Biomek AP96 P250, Presterile with Barrier, 717253 96-Well Riplate-2.2 mL, 43001-0200 Hard-Shell Thin-Wall 96-Well Skirted PCR Plate ABgene 1.2mL 96-Well Storage Plate, AB1127 Assay plate 96 well, Round bottom plate, 3795	Beckman Ritter BioRad ABgene Costar

Results and Discussion

Summary of miRNA yield and quality from exosomes samples extracted on a Biomek workstation.

The average total RNA yield from 25µL of SKOV3 derived exosomes was at 171.63pg/uL+/- 24 analyzed by an Agilent RNA 6000 Pico Chip using Bioanalyzer 2100. Figure 1 (left) shows an example of the miRNA and small RNA profiling (52.4pg/µL with 63% of miRNA) and the middle panel shows an example of total RNA profiling containing small RNA and larger RNA fragments. More abundance of miRNA and small RNA was detected from serum purified exosome samples, (741pg/µL with 77% miRNA) were recovered from 250µL of serum.

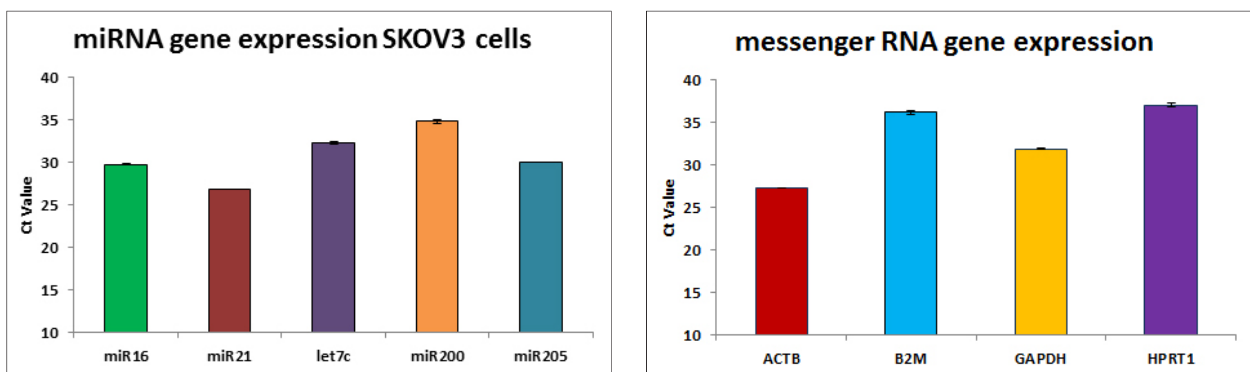
Figure 1: miRNA and RNA profiling from purified exosomes. SKOV3 derived exosomal RNA analyzed on small RNA chip (left), SKVO3 derived exosomal RNA analyzed on RNA Pico Chip (middle). Mouse serum derived exosomal RNA analyzed on the Small RNA chip (right).



Gene expression data demonstrates that miRNA and RNA was successfully extracted from exosome samples using the RNAdvance Cell v2 kit on the Biomek workstation.

5µL of eluted nucleic acid was used for miR16, miR21, let7c, miR200 and miR205 gene expression. The average cycle threshold (Ct) was calculated from triplicate samples. The average Ct values for miR16, miR21, let 7c miR200 and miR205 target gene expression in SKOV3 cells derived exosomes were 29.17+/-0.041, 26.79+/-0.022, 32.23+/-0.17, 34.77+/-0.25 and 30.02+/-0.002 respectively (Figure 2, left). The results showed that miR21was the most highly expressed genes and miR200 was low abundant miRNA in SKOV3 derived exosomes. 5µL of eluted RNA was used for ACTB1, B2M, GAPDH and HPRT house-keeping gene expression to determine whether messenger RNA was successfully extracted from exosomes. The average cycle threshold (Ct) was calculated from triplicate samples. The average Ct values for ACTB, B2M, GAPDH and HPRT1 gene expression were 27.21±0.034, 36.10+/-0.189, 31.78+/-0.07 and 36.98+/-0.19 respectively (Figure 2, right). This result indicates that both miRNA and messenger RNA were successfully extracted from exosomes harvested from a cultured medium using TEI kits and utilizing the RNAdvance Cell v2 kit on Biomek liquid handler.

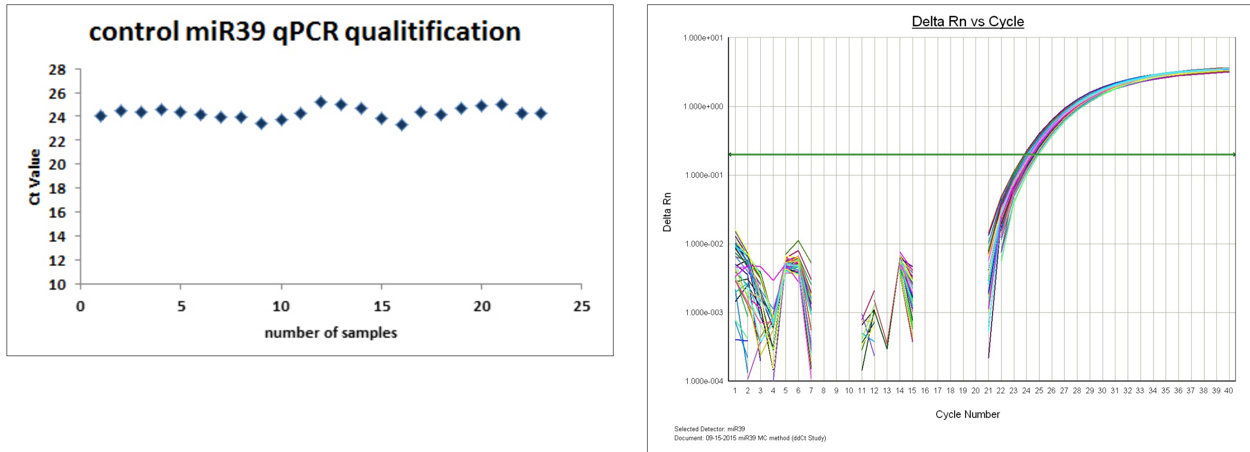
Figure 2: miRNA gene expression from cultured medium of SKOV3 cells (left) and messenger RNA gene expression (right).



Biomek automation extraction showed consistent miRNA recovery with low CV% using control miRNA samples.

To determine the consistency of miRNA recovery using the Biomek extraction method, 1×10^7 copies of miR39 were re-suspended in 1X PBS and used as input samples, and a total of 24 samples were used for qPCR quantification. 1µL of eluted RNA was used for reverse transcription (RT) reaction, and 1µL of cDNA from the RT reaction was used for qPCR amplification. The results showed that the average of 24 samples gave the cycle threshold at 24+/- 0.038 with CV% of 0.2.

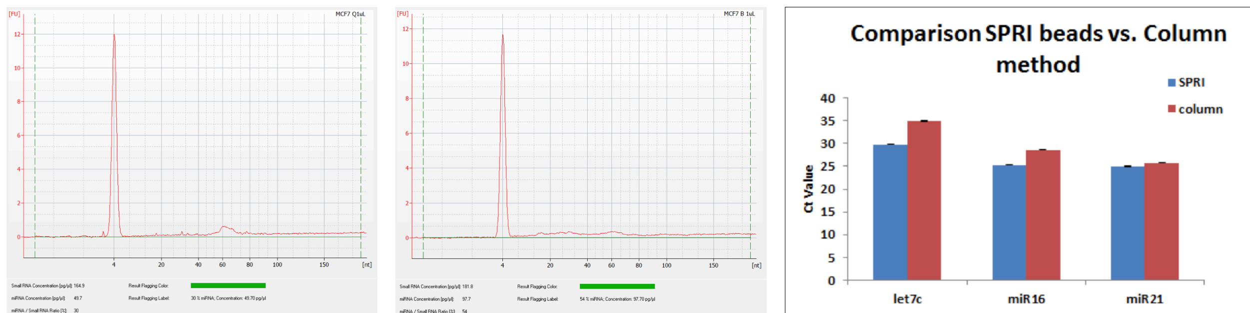
Figure 3: Average of twenty four spiked in miR39 control samples extracted from Biomek (left). The amplification plots of miR39 (right).



The RNAdvance Cell v2 kit yielded higher miRNA recovery as compared to a column purification kit

1×10^6 frozen purified exosomes from the MCF7 breast cancer cell line (EXOP-100A-1, System Biosciences) were used for miRNA and small RNA recovery evaluation using the RNAdvance cell v2 protocol and the miRNeasy serum extraction protocol (Qiagen, 21784). RNA was eluted with 25µL of nuclease-free water in both extractions. The results showed that the RNAdvance Cell v2 kit and the miRNeasy kit gave comparable small RNA yields, however, the RNAdvance Cell v2 kit recovered a higher percentage of miRNA. The RNAdvance Cell v2 kit gave a small RNA concentration of 181.8 pg/µL (54% miRNA) and the column kit gave a small RNA concentration of 164.9 pg/µL (30% miRNA). The data was consistent with the qPCR amplification cycle threshold value that RNAdvance Cell v2 extracted RNA showed a lower cycle threshold when the same amount of starting material was used. For the low expressed let7c gene, the RNAdvance Cell v2 kit gave an average cycle threshold at 29.74+/- 0.03 and the miRNeasy column was at 34.8+/-0.12 cycles. For moderately expressed genes miR16 and miR21, the RNAdvance Cell v2 kit gave 25.26+/-0.027 and 24.87+/-0.102, whereas the miRneasy column kit gave 28.55+/-0.027 and 25.79 +/- respectively. In a second experiment, miRNA expression was performed using A7890 cell-derived exosomes. The expression of miR16 and miR21 using the RNAdvance Cell v2 kits gave 29+/-0.02 and 34.02+/-0.185, whereas the miRNeasy serum kit gave 32.50+/-0.1855 and 32.50+/-0.117 respectively (Data not shown).

Figure 4: small RNA profiling extracted using the miRNeasy kit (left) and the RNAdvance Cell v2 kit (middle). Comparison of miRNA gene expression using the RNAdvance Cell v2 kits vs. the miRNeasey column, 3µL of RNA was used per RT-qPCR reaction (right).



Conclusions

The data from this study shows that the RNAdvance Cell v2 kit gave higher miRNA recovery as compared to the miRNeasy serum kit using microRNA qPCR assays. The RNAdvance Cell v2 Biomek NXP Multi-Channel method is an easy-to-use, automated, user-friendly workstation for nucleic acid extraction that can process from 8 to 96 samples in a 96-well plate format less than 2 hours. It provides a streamlined workflow for downstream assays such as qPCR, micro-array and NGS-RNA sequencing applications.

Acknowledgements

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