Automation of Micro RNA and Total RNA Purification from Plasma Using the Agencourt RNAdvance Blood Kits and Biomek Span-8 Automated Workstation

Bee Na Lee, Ph.D. and John Palys, Beckman Coulter Life Sciences

Extracellular miRNAs that circulate in the bloodstream, known as circulating miRNAs, are remarkably stable. These small non-coding ribonucleic acids (~22 nt) play important roles in gene regulation by binding to and repressing the activity of specific target mRNAs. It is known that tumor cells release miRNAs and RNA into the blood circulation. Profiles of miRNAs and RNA in plasma and serum have been found to be altered in cancer and other disease states. Therefore, circulating miRNAs have been proposed as disease biomarkers that may aid in risk assessment, diagnosis, prognosis, and monitoring of treatment response. This application note describes the purification of miRNA and total RNA from 200µL of serum/plasma samples using the Beckman Coulter SPRI (Solid Phase Reverse Immobilization) magnetic bead based chemistry and a Biomek automated extraction method. The RNAdvance miRNA Blood 96 demonstrated method enables automated purification of total RNA, including miRNA and other small RNAs, from 8–96 samples on a Biomek Span-8 automated workstation. The Biomek automated SPRI method is an easy, high yielding and robust nucleic acid purification process that does not require centrifugation and vacuum filtration steps. Purified nucleic acids are easily eluted from the magnetic beads under aqueous conditions, which provides maximum flexibility for downstream applications. The data shows that Biomek automated extraction yielded in consistent miRNA recovery with low CV% using control miRNA samples. The Biomek demonstrated method successfully extracted miRNAs and messenger RNA with yields comparable to manually extracted samples.

Materials and Methods

200µL of frozen mouse plasma K2-EDTA (LAMPIRE Biological Laboratories, 7304307) or human plasma K2-EDTA was used as testing samples. The miRNA and RNA were extracted using an RNAdvance Blood kit (Beckman Coulter, A35605) according to the instructions for the RNAdvance Blood miRNA protocol (AAG-1021SP07.15-A). For the automated extraction, RNA was extracted using the Beckman Coulter's Agencourt Biomek RNAdvance Blood 96 demonstrated method (A35555 with miRNA enabled method) (Beckman Coulter Life Sciences). Human whole blood was collected in a K2-EDTA anticoagulant tube (Avena Medica) from consenting healthy human adults. Plasma was separated by centrifugation at 1500xg for 10 minutes at 4 degrees C using a Beckman Coulter Allegra X-22R and SX4250 rotor (392187 and 392243) within 2 hours after the blood was collected. The plasma supernatant was transferred and pooled into a nuclease free clean tube, and centrifuged at 3000xg for 15 minutes to remove additional cells and debris prior to miRNA extraction using a Microfuge 18 Centrifuge (Beckman Coulter, 367160). Samples were eluted in 30µL of nuclease free water in the final elution step. Eluted RNA concentration was measured with a plate reader (Molecular Devices, FilterMax F5) using a Ribogreen assay (Life Technologies, R11490). OD260/OD280 ratios were measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). 1 µL of the diluted RNA sample was analyzed on an Agilent small RNA chip (Agilent Technologies, 5067-1548) using the 2100 Bioanalyzer (Agilent Technologies) to determine RNA quality. miRNA (let7c, miR16, miR21, RNU44 and miR155) gene expression was determined using a Taqman microRNA assay (Life Technologies, 4427975 assays ID000379, 000391, 000397, 001006 and 001094 respectively). 3µL of eluted RNA was used for the reverse transcription reaction using the TaqMan micro RNA Reverse Transcription Kit (Life Technologies, 4366596) and 1µL 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 eluted RNA was used for cDNA synthesis using a random primer (Life Technologies, 4368814), and 1µL of the cDNA was used for a 10µL PCR reaction using prime time qPCR assays (Integrated DNA Technologies). The primer

BECKMAN COULTER
Life Sciences

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). Table 1 shows the tools and consumables used for a Biomek Span-8 automated workstation.

Table 1: Tools and consumables needed for 96 samples extraction.

TYPE	QUANTITY	DESCRIPTION	VENDOR
Instrument	1	Biomek Span-8 NX^{P}	Beckman Coulter
Devices	1 1	Orbital Shaker, 379448 Span 8 Passive Wash, 719654	Beckman Coulter Beckman Coulter
ALPS	1	Biomek NX Span-8 4x3 ALP Kit, 989839	Beckman Coulter
Magnet Plate	1	Magnum FLX™ Enhanced Universal Magnet Plate, A000400	ALPAQUA
Reservoirs	1 1 2 2	Reservoir Frame, 372795 Half Reservoir, 534681 Full Reservoir, 372784 Quarter Reservoir, divided by length, 372788	Beckman Coulter Beckman Coulter Beckman Coulter Beckman Coulter
Consumables	8 1 1 1	Span-8 P1000 Tips, Presterile, B01123 Span-8 P250 Tips, Presterile, 379502 Hard-Shell 96-Well Skirted PCR Plate 96-Well Riplate-2.2 mL, 43001-0200	Beckman Coulter Beckman Coulter BioRad Ritter

Results and Discussion

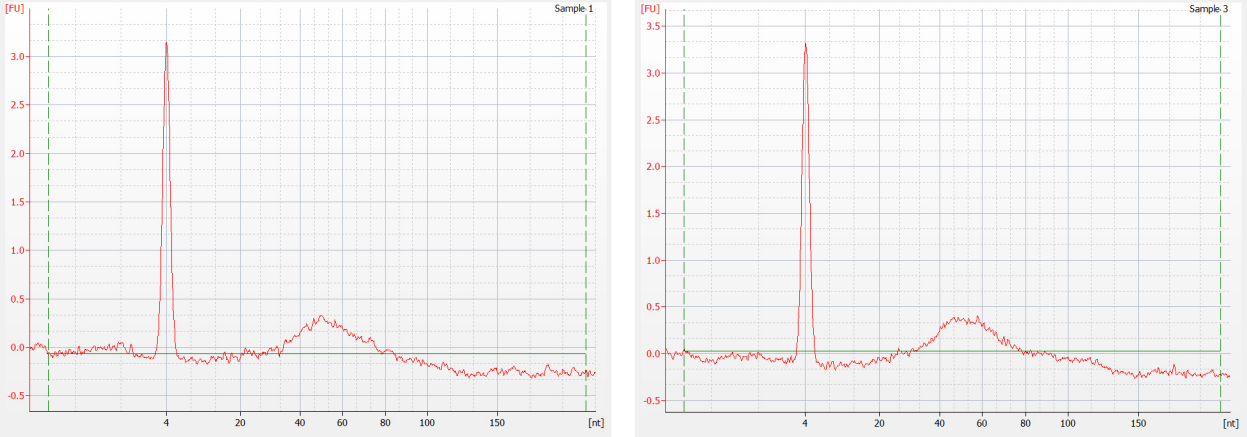
Summary of nucleic acid yields and quality from 48 samples using the Biomek Span-8 automated workstation.

24 replicates of 200µL plasma from two different Biomek runs were used to evaluate nucleic acid yield and quality using the automated RNAdvance Blood extraction method (Beckman Coulter Life Sciences, A35557 modified for miRNA method) that was modified for miRNA extraction. The nucleic acid was eluted in 30 µL of nuclease-free water. The average concentration from 48 samples was at 250 pg/µL with a CV % of 3.8. The calculated average nucleic acid yield per 200µL of plasma was at 7.5ng. The OD260/OD280 ratios ranged from 1.0-2.1 with an average of 1.6. Relatively low ratios of OD 260/OD280 are due to low abundance of nucleic acids in the samples. Figure 1 shows the examples of RNA profiles.

Table 2: The average concentration and yield from a total of two batches of 24 replicate plasma samples.

AVERAGE CONC. PER 200µL (pg/µL)+/- CV%	AVERAGE YIELD (ng) PER 200µL	ELUTION VOLUME (µL)
252+/-3.7%	7.6ng	30
248+/-4%	7.44ng	30

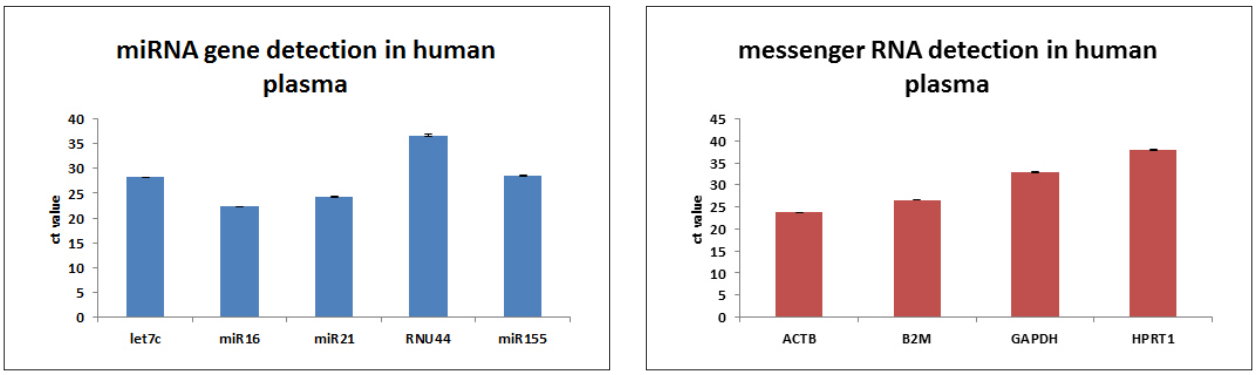
Figure 1: Example of total RNA Profiling on gel view and electropherograms.



MiRNA and messenger RNA gene expression data demonstrate that circulating miRNA and RNA were successfully extracted using the Biomek Span-8 automated workstation.

3µL of eluted RNA was used for let7c, miR16, miR21, RNU44 and miR155 gene expression. The average Ct values for let7c, miR16, miR21, RNU44 and miR155 target gene expression human plasma were 28.167+/-0.026, 22.278+/-0.02, 24.244+/-0.028, 36.577+/-0.227 and 28.52+/-0.035 respectively (Figure 2, left). This result indicates that miRNA was successfully extracted from plasma utilizing the RNAdvance Blood kit on the Biomek liquid handler. The data also showed that miR16 was the most abundant miRNA in circulating blood. For messenger RNA gene expression, ACTB, B2M, GAPDH and HPRT1 genes were used for evaluation. The average Ct values for ACTB, B2M, GAPDH and HPRT1 gene expression were 23.752+/-0.028, 26.621+/-0.021, 32.859+/-0.146 and 38+/-0.181 respectively (Figure 2, right). ACTB was the most abundant and HPRT was the least abundant found in these samples. The controls with no template showed no amplification, indicating that the amplification resulted from miRNA or messenger RNA alone (data not shown).

Figure 2: miRNA gene expression (left) and messenger gene expression (right) from 200µL of human plasma.

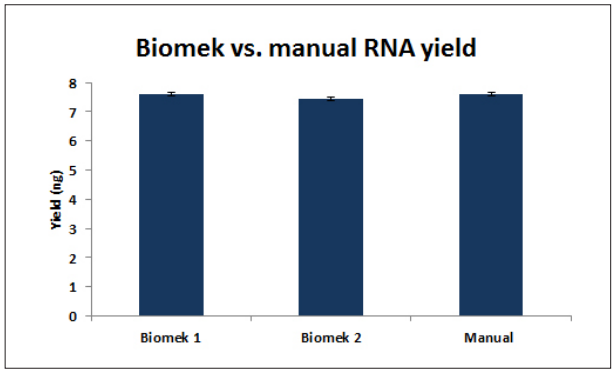


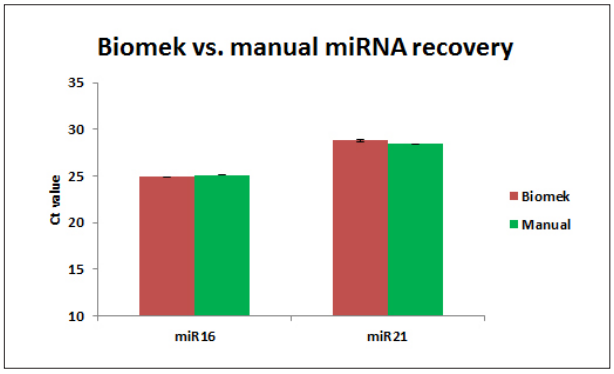
The Biomek automated extraction resulted in comparable RNA yields and miRNA recovery as compared to manual extraction.

To compare the RNA yield between manual extraction and Biomek automated extraction, the average RNA yield was calculated from three batches of plasma. Figure 3 (left) shows that automated extraction resulted in comparable RNA yields (Biomek 1, 7.6ng, Biomek 2=7.44ng, manual=7.6ng). 750pg of total RNA was used to determine miR16 and miR21 gene expression from either manual or Biomek automation purified RNA samples. The average Ct value for miR16 and miR21 gene expression from the Biomek-extracted samples was 25.11+/- 0.0385 and 28.82+/-0.146

respectively, whereas for the manually-extracted samples showed a Ct value of 24.913+/-0.0245 and 28.4+/- 0.042 respectively. The result indicates that both extraction methods yielded in comparable miRNA recovery (Figure 3, right). The minus RT and controls with no template showed no amplification, indicating that the amplification resulted from miRNA and mRNA alone (data not shown).

Figure 3 left: Average RNA yields for Biomek automation and manual extraction methods. The Y axis represents RNA yield (ng). The X axis represents two different Biomek extracted samples and manually-extracted samples. Biomek 1 (n=24), Biomek 2 (n=24), manual (n=8). **Figure 3 right:** Comparison of miRNA recovery between Biomek vs. manually extracted samples.





Conclusions

The data from this study show that Beckman Coulter's RNAdvance Blood Kit can be used for miRNA and RNA extraction from plasma or serum (AAG-1025APP07.15-A). The RNAdvance Blood 96 Biomek Span-8 demonstrated method is an easy-to-use, robust, fully automated, user-friendly workflow for nucleic acid extraction from 200-300µL of plasma/serum or 400µL of PAXgene preserved blood samples. It can process from 8 to 96 samples in a 96-well plate format in about 2.5-3 hours. It provides a streamlined workflow for downstream assays such as qPCR, micro-array and NGS-RNA sequencing applications.