CIRCULATING CELL FREE DNA AND MIRNA EXTRACTION FROM PLASMA USING BECKMAN COULTER'S Agencourt RNAdvance Blood Kits

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Summary

Tumor cells release circulating cell free DNA (cfDNA) into the blood and it is believed that elevation of these cfDNAs in the plasma of patients may be associated with malignancy. Advanced technologies have made it possible to identify specific genetic alterations and to detect rare mutations in a background of wild type sequences from blood samples, known as a non-invasive liquid biopsy. Analysis of blood samples for the presence of circulating tumor cells (CTCs) or cell free circulating tumor DNA (ctDNA) can be performed repeatedly, and might allow real-time monitoring to detect the earliest stages of tumor growth and selection of cancer therapies for treatment resistance in individual patients.

This technical note describes the method development for purification of cfDNA from 200µL-300µL of plasma using Beckman Coulter's Agencourt RNAdvance Blood kit. Beckman Coulter's SPRI (Solid Phase Reverse Immobilization) paramagnetic bead-based chemistry provides an easy, rapid, high yielding, robust and automation-friendly nucleic acid purification procedure that does not require vortexing, centrifugation or filtration steps. The data shows that cfDNA was successfully extracted and detected from human plasma samples using qPCR assays. Both cfDNA and miRNA can be extracted from the same protocol.

Materials and Methods

Human whole blood was collected in a K2-EDTA anticoagulant tube (Avena Medica) from consenting adults. Plasma was prepared by centrifugation at 1500xg for 10 minutes at 4°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 16,000xg for 15 minutes to prepare supernatant plasma without cell debris and with minimized genomic DNA contamination using a Microfuge 18 Centrifuge (Beckman Coulter, 367160). 200µL of frozen human plasma K2-EDTA was digested with 300µL lysis buffer and 20µL of proteinase K for 30 minutes by shaking at 1000 rpm at room temperature. cfDNA was extracted using an RNAdvance Blood kit (Beckman Coulter, A35605) and the cfDNA supplemental protocol (reference #1, AAG-1256SP11.15-A) without RNase treatment. Nucleic acids were eluted in 30µL of nuclease free water in the final elution step. Eluted nucleic acid 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). A control non-human synthetic cel-miR39 (Qiagen, 219610) was spiked into the bovine plasma to determine the optimal binding and washing conditions for small nucleic acid recovery using the RNAdvance Blood plasma supplemental protocol (reference #1 AAG-1256SP11.15-A). The miRNA recovery was measured using a quantitative Taqman qPCR assay (Life Technologies, 4427975, assay ID 000200). qPCR assays for miRNA gene expression were performed as described in reference #2(AAG-1025APP07.15-A). Primers for DNA detection were designed from exon sequences using IDT primer design tool, Primerquest. http://www.idtdna.com/Primerquest/Home/Index, the primer sequences were checked for SNPs and non-specific bindings. 5µL of eluates was used for qPCR amplification in a 10µL reaction using Taqman Universal Master Mix II (Life Technologies, 4440038). Primer sequences used for this study are shown in table 1

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Genes	Primer 1	Primer 2	Probe
ACTB1	GGAAATCGTGCGTGACATTAAG	AGCTCGTAGCTCTTCTCCA	CTGGACTTCGAGCAAGAGATGGCC
AKT1	GGCTGTGCCTCAGGTTG	CACAATCTCAGCGCCATAGAA	AGCTGTTCTTCCACCTGTCCCG
B2M	TGA TGT ATC TGA GCA GGT TGC	GCT TTG AGT GCA AGA GAT TGA AG	TA GGA GGG CT GGC AAC TTA GAG GT
EGFR	CTGGACCTTGAGGGATTGTTT	AACTGCGTGAGCTTGTTACT	TTTCTTCCAGTTTGCCAAGGCACG
HPRT1	TGT CAG TTG CTG CAT TCC T	TCA CTC AAT AGT GCT GTG GTTA TA	AA CAA CAA TC CGC CCA AAG GGA AC
KRAS	CAGACTGTGTTTCTCCCTTCTC	CTCATGTACTGGTCCCTCATTG	TCGACACAGCAGGTCAAGAGGAGTA
P53	ACAATGGCTCCTGGTTGTAG	AGCATCTGTATCAGGCAAAGT	TTAAAGGACCAGACCAGCTTTCAA

Results and Discussion

The RNAdvance Blood kit protocol was developed for PAXgene preserved blood total RNA purification. The binding conditions were optimized in capturing nucleic acids fragments that are larger than 100bp and any smaller fragments were removed during washing steps. In order to capture smaller nucleic acid fragments, the binding conditions were modified and optimized for 22nts using experimental design as described in reference #3(B2012-13440). To determine the DNA recovery efficiency, 1µg of puc18 HaeIII digested DNA fragments (Sigma, D6293) was spiked in 200µL, 300µL or 400µL of bovine plasma as testing samples and eluted with 40µL of nuclease free water. An estimated yield of 25ng/uL would be expected if recovery reaches 100%. Figure 1 shows the recovery of the DNA measured using a 4% EX-gel and an E-Gel agarose electrophoresis apparatus (Life Technologies, G401004). The results showed that the 200µL (lane 3) and 300 uL (lane 4) samples yielded similar recovery as compared to the control, unpurified 25ng/uL (lane 2). However, less of the 80bp fragment was recovered in the 400µL plasma (lane 5). Therefore, the protocol is recommended for up to 300µL extraction.

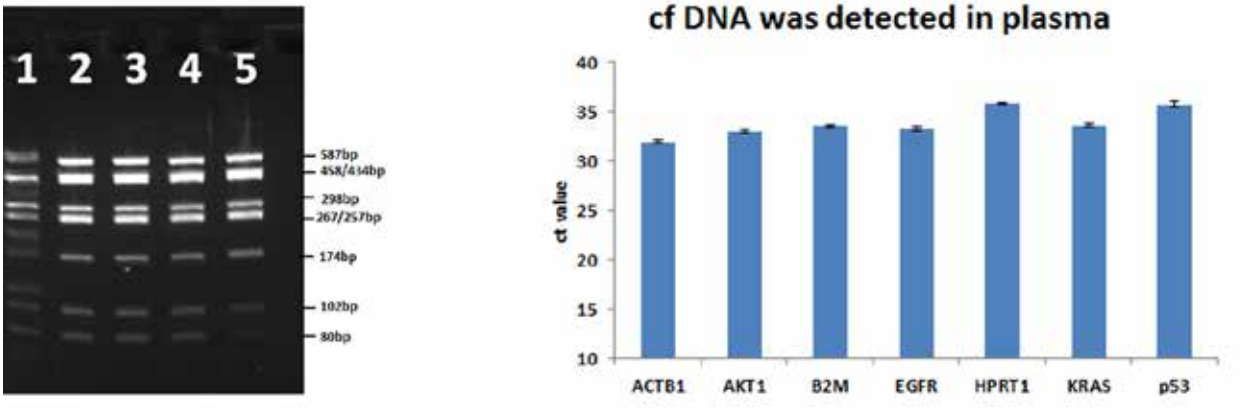

Figure 1 left. 1µL of sample was loaded in each lane. Lane 1 was the 10bp DNA size marker ladder, lane 2 was the control (25ng of unpurified puc18 HaeIII digested DNA), lanes 3-5 were loaded with 1µL the purified puc18 HaeIII digested DNA from 200µL, 300µLand 400µL bovine plasma respectively.

Figure 1 right. cycle threshold values of cfDNA amplified from 1ng of nucleic acids extracted from 200µL of human plasma.

qPCR assay demonstrates that circulating DNA was successfully extracted from human plasma samples.

To determine whether circulating cfDNA was successfully extracted from human plasma, specific primers and probes were designed from the exon sequences of the following genes: ACTB, AKT1, B2M, EGFR, HPRT1, KRAS and P53. 5µL of eluted samples were used for the qPCR reactions. The average Ct values for ACTB1, AKT1,B2M, EGFR, HPRT1, KRAS and P53 were 31.88+/-0.153, 32.95+/-0.112, 33.50+/-0.155, 33.19+/-0.23, 35.77+/-0.078, 33.55+/-0.11 and 35.70+/-0.27 respectively (Figure 1, right). The results indicate that cfDNA was successfully extracted from human plasma using the RNAdvance Blood kits.

RNAdvance Blood kit modified protocol can be used for both miRNA and cfDNA extraction

Nucleic acid eluates from the same plasma samples were used for miRNA gene expression using specific miRNA primers and probes. Figure 2 shows that the miR16 and let7c amplification efficiency from two replicates. 3µL of eluates was used for the reverse transcription reaction and 1µL of cDNA was used for PCR reaction in triplicates. The average Ct values for miR16 were 24.89+/-0.05 and 25+/-0.013; and for let7c were 35.15+/-0.268 and 35.18+/-0.138. The data indicates that the same protocol can be used for circulating miRNA and cfDNA extraction.

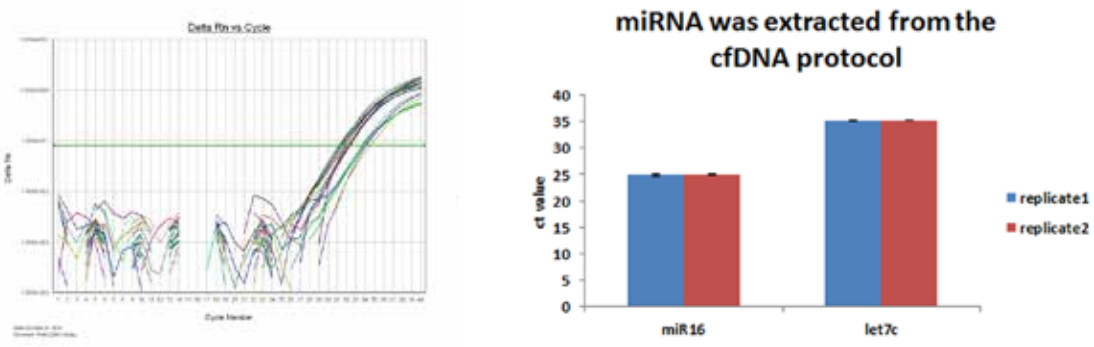

Figure 2 left. amplification plots from seven different genes using exon specific sequences.

Figure 2 right. replicates of RT-qPCR miRNA assay using miR16 and let7c specific primer and probes. 750pg of nucleic acids extracted from 200µL of human plasma was used per reaction.

Conclusions

The data from this study shows that Beckman's RNAdvance Blood Kit can be used for circulating miRNA and cfDNA extraction from plasma. The magnetic bead-based extraction protocol provides scalable throughput and it is automation-friendly. The Biomek RNAdvance Blood 96 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 (reference #4, AAG-1251APP11.15). 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.

References

1. Supplemental Protocol for free circulating DNA and miRNA/RNA isolation from 200-300µL of plasma and serum (AAG-1256SP11.15-A).
2. MicroRNA and RNA extraction from plasma and serum using Beckman Coulter's Agencourt RNAdvance Blood kits (AAG-1025APP07.15-A).
3. Highly-efficient miRNA Isolation using the Agencourt FormaPure and RNAdvance Cell v2 Kits and Biomek Automated Extraction Methods (B2012-13440).
4. Automation of Micro RNA and Total RNA Purification from plasma using the Agencourt RNAdvance Blood Kits and Biomek Span-8 automated workstation (AAG-1251APP11.15-A).