Small RNA Sequencing of Plasma Exosomes Collected on Heparin: Quality and Quantity Challenges

Application Note 99

Keywords

+ NGS
+ Small RNA-seq
+ Exosomes
+ Heparin
+ RNA
+ DNA
+ Plasma
+ Serum
+ Exosomal RNA
+ Cell Free
+ Library Prep
+ Liquid Biopsy
+ Blood

INTRODUCTION

Several blood collection tubes with anticoagulants are used to separate blood and collect plasma. Potassium EDTA, heparin and citrate are the most commonly used type of blood collection devices[1]. The selection of appropriate blood collection tube is important as some of these anticoagulants may interfere with the downstream processing of the sample. Heparin is one such anticoagulant, which is co-purified during RNA/DNA extractions, and it interferes with the reverse transcriptase as well as the Taq DNA polymerase[2,3]. This makes the RNA/DNA extracted from plasma collected using heparin tubes unusable for PCR based applications including sequencing. Treating the extracted DNA/RNA with Heparinase can degrade the heparin, which allows the DNA/RNA to be able to be used for further downstream processing such as sequencing[3].

Sequencing of the RNA extracted from the bio-fluids has gained interest in the recent years due to its application as non-invasive biomarker to an underlying disease condition. Exosomal small RNA sequencing gives even better understanding of the disease conditions as the RNA profiles are more specific to cell-cell communication and transmission of disease[4]. However, extraction of Exosomal RNA can yield RNA in the pico gram range. The presence of heparin in this minute amount of exosomal RNA pose an obstacle in molecular downstream applications, including NGS. Combining efficient sample extraction together with robust heparin removal can render these samples sequencable. Here, we present a study on successful small RNA sequencing from RNA extracted from plasma exosomes collected on heparin.

MATERIALS AND METHODS

Plasma Preparation and RNA Extraction

Plasma from three donors separated from whole blood collected on sodium heparin were purchased from Zenbio Inc. (Cat# SER-PLE-10ML). The plasma was aliquot in to 500uL volumes and were stored at -70 °C upon arrival until further use. For exosomal RNA extraction, first, the plasma was thawed and centrifuged at 425g for 2 minutes to obtain cell free plasma. Exosomal RNA was extracted from all the three donor samples using Norgen's Plasma/Serum Exosome Purification and RNA Isolation Mini Kit (Cat. 58300) and the RNA analyzed on an RNA 6000 Pico chip using Agilent 2100 Bioanalyzer System (Agilent Technologies, USA).

Removal of Heparin from Exosomal RNA

Heparinase treatment method as described by Kondratov et. al., was used where, 20µL RNA sample was mixed with 20µL of Heparinase working solution (0.085 IU/mL of Heparinase I (Sigma-Aldrich; Cat# H2519), 2000 units/mL of RiboLock RNase Inhibitor (Life Technologies; Cat# EO0381), 10 mmol/L Tris HCl pH 7.5, 2 mmol/L CaCl2, 25 mmol/L NaCl) and incubated at 25°C for 3 h3. The RNA was stored at -80 °C until further use and were used directly for small RNA sequencing.

Small RNA Library Preparation and Sequencing

Small RNA library was constructed from all the exosomal RNA samples using Norgen's Small RNA Library Prep Kit for Illumina (Cat. 63600). The libraries were quantified on the High Sensitivity DNA Analysis Chip using the Agilent 2100 Bioanalyzer System (Agilent Technologies, USA). All the libraries were diluted to 4nM concentration, pooled and sequenced on the Illumina NextSeq 550 platform using the NextSeq 500/550 High Output Kit v2.5 (75 cycles). Fifty cycles were used for the sequencing run.

Reads Processing and Analysis

Fastqc (version 0.11.9) and Multiqc (version 1.12) were used to perform quality check of sequences. Analysis of the small RNA sequences was done using exceRpt Pipeline (Version 4.6.2) with the hg38 database (GRCh38.p13). The raw read counts obtained were normalized by calculating the reads per million (RPM) values.

RESULTS AND DISCUSSION

With the increase in the use of the liquid biopsies as a non-invasive tool for disease diagnosis and progression, the demand for the use of plasma collected on multiple types of devices is increasing. The downstream processing of the nucleic acids, especially RNA from the plasma separated on sodium heparin tubes has shown importance as heparin tubes are one of the commonly used blood collection tubes. The current study was focused on the small RNA sequencing of heparinase treated exosomal RNA extracted from plasma collected on heparin tubes.

RNA Characteristics, Read Alignment and Biotype Distribution

As per the Agilent 2100 Bioanalyzer data, the average exosomal RNA yield obtained from 200µl heparin plasma was 6.68 ng at an average concentration of 323 pg/µl (min - 298pg/µl; max – 351 pg/µl) and RIN values for each sample was between 2.5 and 2.6. The typical RNA yield from exosomes was similar to the RNA yield reported by Huang and group[5]. Amongst the three samples, a total of 41.5 million reads were generated, out of which 9.17 million (22.10%) were short reads, 0.76 million (1.84%) were without adapter, 0.28 (0.67%) million were rRNA, 0.07 million (0.18%) were UniVec contaminants and 31.2 million (75.19%) reads passed initial QC and were able to be used for alignment **(Figure 1)**.

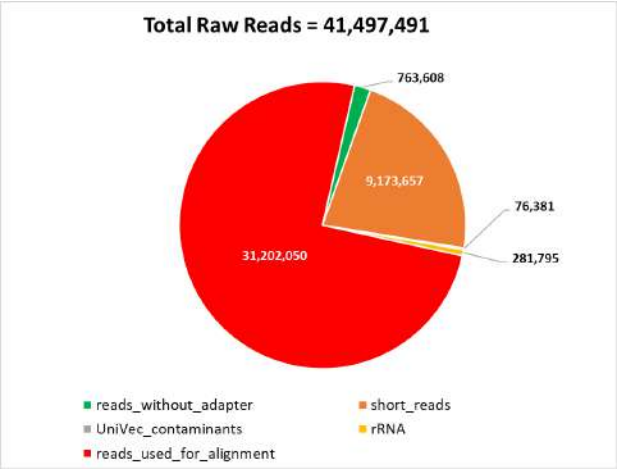

Figure 1. Distribution of input reads alignment from three exosomal RNA samples.

The average reads from the three-heparin plasma samples obtained after adapter trimming and quality filtering was 10.51 ± 1.76 million and that was comparable with the same study[5]. Approximately 0.9% reads were mapped to rRNA sequences and were thus excluded from further analysis. The highest number of reads were assigned to tRNA (37.1%), which was lower as compared to what was reported in a serum exosome study by Tang *et. al.,* where they found 54.3% reads mapping to tRNA. The same group was able to map only 4.3% reads to the miRNA, which is similar to what we observed in our study (6%)[6].

The average number of reads that were mappable to the human genome were 1.49 ± 0.29 million. Out of all the reads mapped to genome, the average microRNA reads were 6.5%, 37.1% tRNA, 2.4% circular RNA, 0.15% lincRNA, 0.32% piRNA. The counts for snRNA, snoRNA, and scaRNA were very low while there were 0% reads for MtRNA and sRNA. The distribution of the RNA species with average reads is shown in **figure 2**.. The total number of miRNA detected was in the range of 146-204. Furthermore the analysis for miRNA abundance revealed that hsa-miR-26a-5p, hsa-miR-486-5p, hsa-miR-191-5p, hsa-miR-92a-3p and has-let-7f-5p were the most abundant small RNA species which was similar to the miRNA abundance in plasma samples as reported by El-Mogy and group in 2018[7].

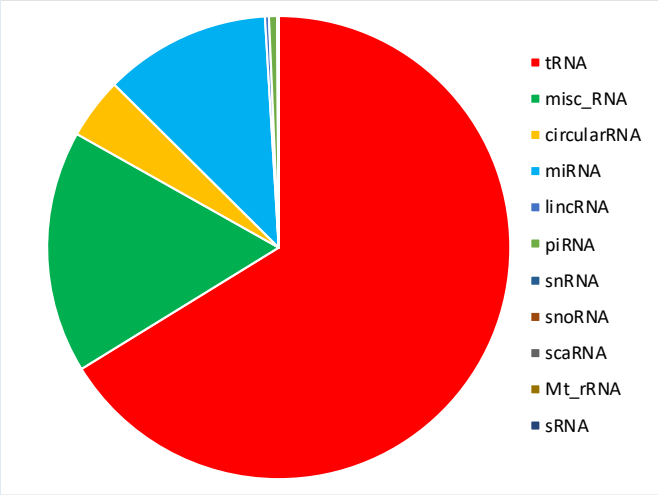

Figure 2. Average biotype distribution of the exosomal small RNA.

CONCLUSION

1. Heparinase treatment of the exosomal RNA extracted from the plasma separated using heparin tubes can be used to give successful construction of small RNA sequencing libraries and subsequent sequencing.

2. The raw reads and the sample biotype is similar to the profile of exosomal RNA extracted from non-heparin base plasma samples.

3. The abundance of the miRNA obtained is similar to the miRNA abundance of the RNA extracted from non-heparin based plasma samples.

REFERENCES

1. Lam NY, Rainer TH, Chiu RW, Lo YD. EDTA is a better anticoagulant than heparin or citrate for delayed blood processing for plasma DNA analysis. Clinical chemistry. 2004 Jan 1;50(1):256-7.
2. Johnson ML, Navanukraw C, Grazul-Bilska AT, Reynolds LP, Redmer DA. Heparinase treatment of RNA before quantitative real-time RT-PCR. Biotechniques. 2003 Dec;35(6):1140-4.
3. Kondratov K, Kurapeev D, Popov M, Sidorova M, Minasian S, Galagudza M, Kostareva A, Fedorov A. Heparinase treatment of heparin-contaminated plasma from coronary artery bypass grafting patients enables reliable quantification of microRNAs. Biomolecular detection and quantification. 2016 Jun 1;8:9-14.
4. Narang P, Shah M, Beljanski V. Exosomal RNAs in diagnosis and therapies. Non-coding RNA Research. 2022 Mar 1;7(1):7-15.
5. Huang X, Yuan T, Tschannen M, Sun Z, Jacob H, Du M, Liang M, Dittmar RL, Liu Y, Liang M, Kohli M. Characterization of human plasma-derived exosomal RNAs by deep sequencing. BMC genomics. 2013 Dec;14(1):1-4.
6. Tang YT, Huang YY, Zheng L, Qin SH, Xu XP, An TX, Xu Y, Wu YS, Hu XM, Ping BH, Wang Q. Comparison of isolation methods of exosomes and exosomal RNA from cell culture medium and serum. International journal of molecular medicine. 2017 Sep 1;40(3):834-44.
7. El-Mogy M, Lam B, Haj-Ahmad TA, McGowan S, Yu D, Nosal L, Rghei N, Roberts P, Haj-Ahmad Y. Diversity and signature of small RNA in different bodily fluids using next generation sequencing. BMC genomics. 2018 Dec;19(1):1-24.

RELATED PRODUCTS

Product	Cat. No
Plasma/Serum Exosome Purification and RNA Isolation Mini Kit	58300
Small RNA Library Prep Kit for Illumina	63600

YOUR CUSTOM NGS WORKFLOW STARTS WITH NORGEN

- **STEP 1:** Book your FREE Consultation
- **STEP 2:** Sample Submission & Shipping
- **STEP 3:** Sample Isolation
- **STEP 4:** Quality Control
- **STEP 5:** Library Preparation
- **STEP 6:** Sequencing
- **STEP 7:** Bioinformatics Analysis

Book Your Free Consultation Today

Discover more by scanning the QR code or visiting norgenbiotek.com/ngs

SCAN ME WITH YOUR SMART PHONE

ISO 15189:2012 CERTIFIED QUALITY · ISO 13485:2016 CERTIFIED QUALITY · ISO 9001:2015 CERTIFIED QUALITY

Norgen Biotek Corp.
3430 Schmon Parkway,
Thorold, ON, Canada, L2V 4Y6

E info@norgenbiotek.com
T 1 866 NORGENB
norgenbiotek.com