EXTRAClean RNA Isolation Technology Elevates Small RNA Sequencing Reads Obtained from Plasma Exosomal RNA

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INTRODUCTION

Exosomes are primarily known to be involved in communication within various systems in the human body as they are normally secreted by immune cells, neuronal cells, cardiomyocytes, stem cells and other cells [1]. They can be found in almost all bodily fluids including plasma, serum, urine, saliva, etc., and may carry DNA, RNA, lipids, proteins, and metabolites based on the cell where they originated [2,3]. Apart from performing normal physiological functions, exosomes are also involved in disease progression as well as the pathological development of several diseases such as heart failure, neurodegenerative diseases, liver diseases, and cancer [1, 4-7].

RNA being more indicative of the disease progression and transmission, sequencing of exosomal RNA provides an in-depth understanding of current underlying disease status [8, 9]. Exosomal mRNA, miRNA, circRNA, and lncRNA, that are known to regulate the biological processes of the cells, are also known to be involved in disease progression of sarcomas, pancreatic and colon cancers, gastric tumors, prostate cancer, and lung cancer [9].

Moreover, exosomes have a very low amount of RNA present in them, which is reflected in the obtained RNA yields. Sequencing of these low RNA amounts is challenging due to the limited RNA amount and the required high purity to obtain the best sequencing outcomes. In the current study, we present our EXTRAClean technology that provides very high-quality and pure RNA from plasma exosomes that significantly enhances NGS performance.

Materials and Methods

PLASMA PREPARATION AND RNA ISOLATION

Blood was collected from three donors to prepare plasma in EDTA tubes (BD, Cat# 366643). Plasma was separated and was further processed to remove cells by centrifuging at 2500xg for 10 minutes. Cell-free plasma was transferred to a fresh tube and was stored at -80°C until further use. Intact Exosomes were purified from 0.2 mL plasma using Plasma/Serum Exosome Purification Mini Kit (Norgen Biotek Corp., Cat# 57400). Extracted exosomes were further processed to extract exosomal RNA using Exosomal RNA Isolation Kit (Norgen Biotek Corp., Cat# 58000) and EXTRAClean Exosomal RNA Isolation Kit (Norgen Biotek Corp., Cat# 72800).

EXTRAClean Exosomal RNA Isolation Kit (Norgen Biotek Corp., Cat# 72800).

SMALL RNA LIBRARY PREPARATION AND SEQUENCING

Libraries were prepared using Small RNA Library Prep Kit for Illumina (Norgen Biotek Corp., Cat# 63600) and were quantified using High Sensitivity DNA Assay run on the Agilent 2100 Bioanalyzer System (Agilent Technologies, Cat# 5067-4626). The libraries were further diluted to 4 nM concentration and were pooled in an equimolar ratio. Pooled libraries were sequenced on the Illumina NextSeq 550 platform using the NextSeq 500/550 High Output Kit v2.5 (75 cycles) for 51 cycles (Illumina, Cat# 20024906).

READS PROCESSING AND ANALYSIS

Obtained reads were checked for quality using Fastqc (version 0.11.9). Qualified small RNA sequences were analyzed by exceRpt Pipeline (Version 4.6.2) by aligning to the hg38 database (GRCh38.p13). Mapped read counts were normalized by calculating the reads per million (RPM) values.

Results and Discussion

The average exosomal RNA yield obtained from 200µL EDTA plasma using the EXTRAClean version was 3.9 ng (Avg. conc. 78.7 pg/µL) while the using the control version was 11.2 ng (Avg. conc. 224.0 pg/µL). The RIN values of RNA obtained from both versions were low (avg. RIN; EXTRAClean – 1.9, Control – 1.0). There was a reduction in the RNA yield obtained using the EXTRAClean kits as compared to the control kits as the EXTRAClean kit is optimized to isolate higher quality RNA with the least amount of background RNA and thus represents the true exosomal RNA.

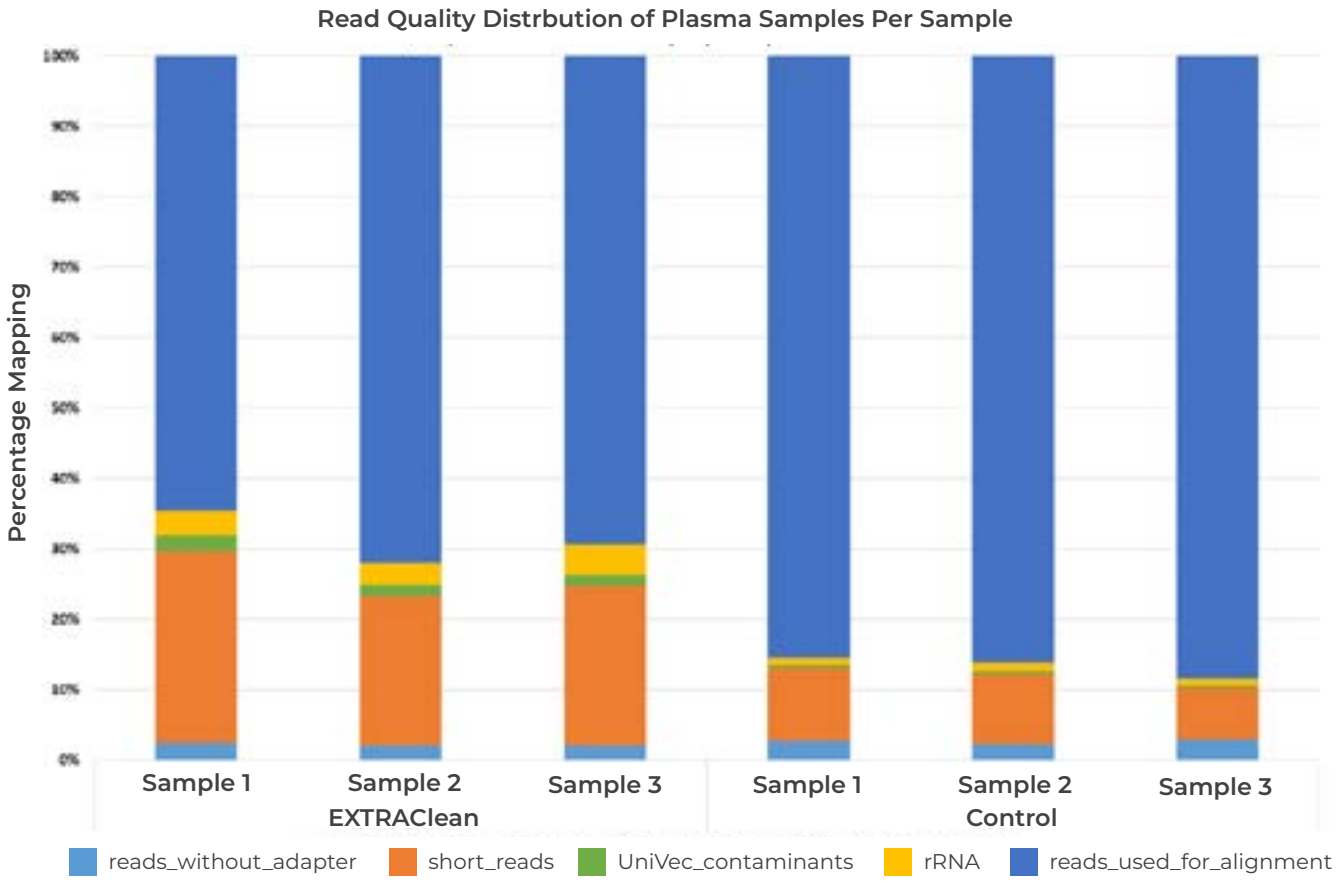

Figure 1. Average read quality distribution relative to raw reads of small RNA sequencing of exosomal RNA extracted from three plasma samples using EXTRAClean Exosomal RNA Isolation Kit (Norgen Biotek Corp., Cat# 72800) and Exosomal RNA Isolation Kit (Norgen Biotek Corp., Cat# 58000). Calculations were relative to raw reads.

In case of EXTRAClean kits, a total of 15.1 million reads were generated, out of which 0.3 million (2.24%) were reads without adapter, 3.5 million were short reads (23.64%), 0.5 million (3.62%) were reads mapped to rRNA and 0.27 million reads (1.83%) were UniVec contaminants. Reads passing QC were 10.3 million reads (68.64%) and were further used for alignment **(Figure 1**). On the other hand, the control kit generated a total of 30.6 million reads. Amongst these reads, 0.8 million (2.63%) were reads without adapters, 2.8 million (9.19%) were short reads, 0.3 million (1.1%) were rRNA reads and 0.1 million reads (0.43%) were UniVec contaminants. The remaining 26.5 million (86.62%) were reads that passed QC parameters **(Figure 1**).

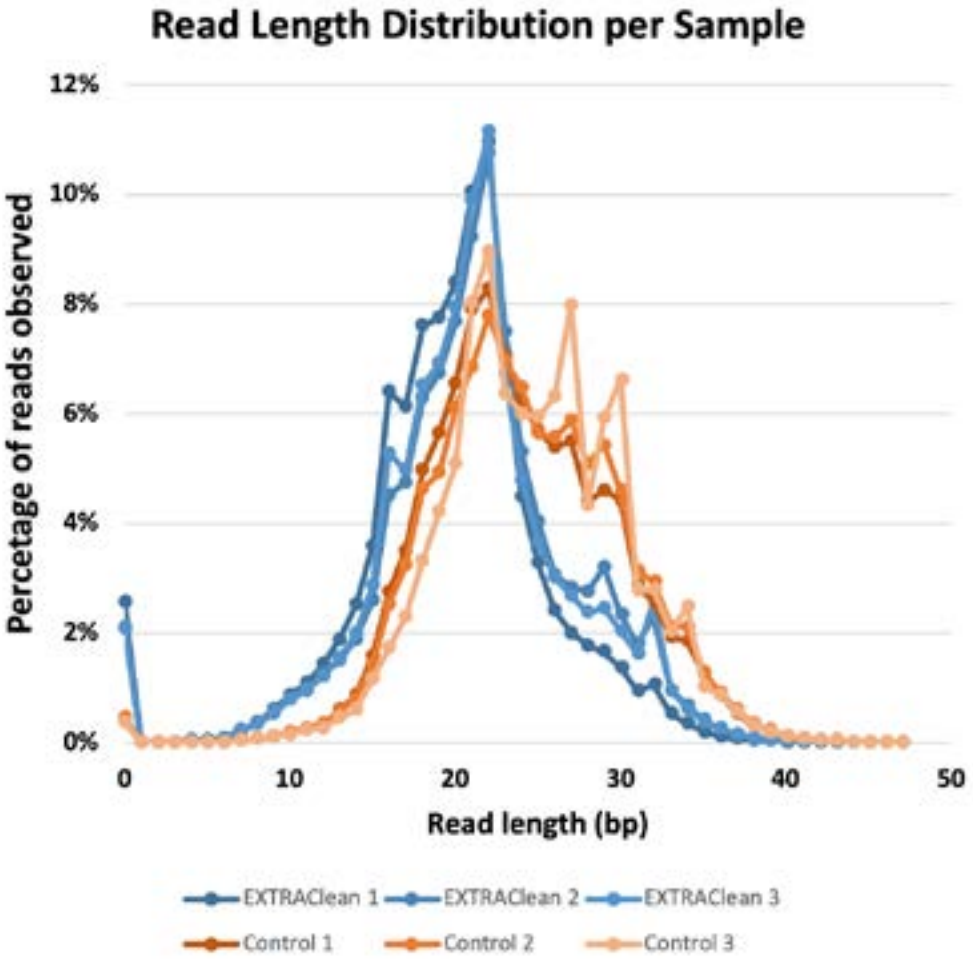

Figure 2. Read length distribution obtained from sequencing of exosomal RNA extracted from three plasma samples using EXTRAClean Exosomal RNA Isolation Kit (Norgen Biotek Corp., Cat# 72800) and Exosomal RNA Isolation Kit (Norgen Biotek Corp., Cat# 58000). Calculations were relative to raw reads.

Based on the above-mentioned details, the EXTRAClean kit generated a larger proportion of reads shorter than 18 nucleotides as compared to the control kit. The EXTRAClean kits are more enriched in smaller sizes of RNA as can be observed in Figure 2, the graph showing the read size distribution of RNA extracted using the EXTRAClean kit is more shifted towards the smaller sizes and enriched near 22bp which is attributed to miRNA. This enrichment was also observed in the miRNA analysis, as the EXTRAClean kits could detect 168 miRNAs while the control kit could detect 128 miRNAs. Moreover, on performing Novel miRNA prediction using miRDeep2 at default parameters and a cutoff score of 5, it was found that the EXTRAClean kits discovered significantly higher (p=0.0070) novel miRNA as compared to the control kit **(Table 1)**.

Volume	Exosome Purification Kit	Sample Number	Number of Novel miRNA	Average	Standard Deviation
0.2 mL	EXTRAClean	1	40	38.67	1.5
		2	39		
		3	37		
	Control	1	14	18.67	4.0
		2	21		
		3	21		

Table 1. Results showing comparison of Novel miRNA prediction between EXTRAClean and control kit using the miRDeep2 pipeline. Predicted miRNA with score higher than a miRDeep2 score of 5 was used.

Furthermore, as compared to the control kit, the EXTRAClean kit was able to capture approximately 7% more reads that were mapped to the genome. This increase brought the total genome mapped reads to 2.4 million (16.23%), while that of the control kit was 2.8 million (9.14%), reflecting about 78% increase in genome mapped reads. **(Figure 3)**. Moreover, the control kit (23.7 million; 77.48%) showed more unmapped reads as compared to the EXTRAClean format (7.8 million; 52.4%) **(Figure 3).**

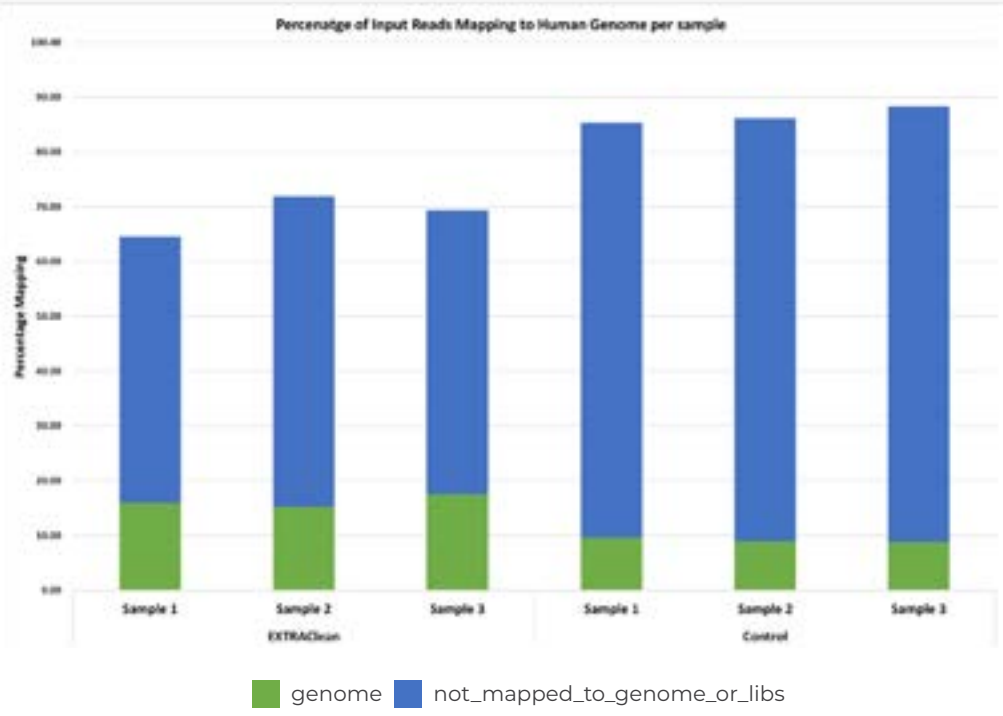

Figure 3. Genome mapping relative to input reads obtained from small RNA sequencing of exosomal RNA extracted from plasma three samples using EXTRAClean Exosomal RNA Isolation Kit (Norgen Biotek Corp., Cat# 72800) and Exosomal RNA Isolation Kit (Norgen Biotek Corp., Cat# 58000). (Norgen Biotek Corp., Cat# 72800) and Exosomal RNA Isolation Kit (Norgen Biotek Corp., Cat# 58000).

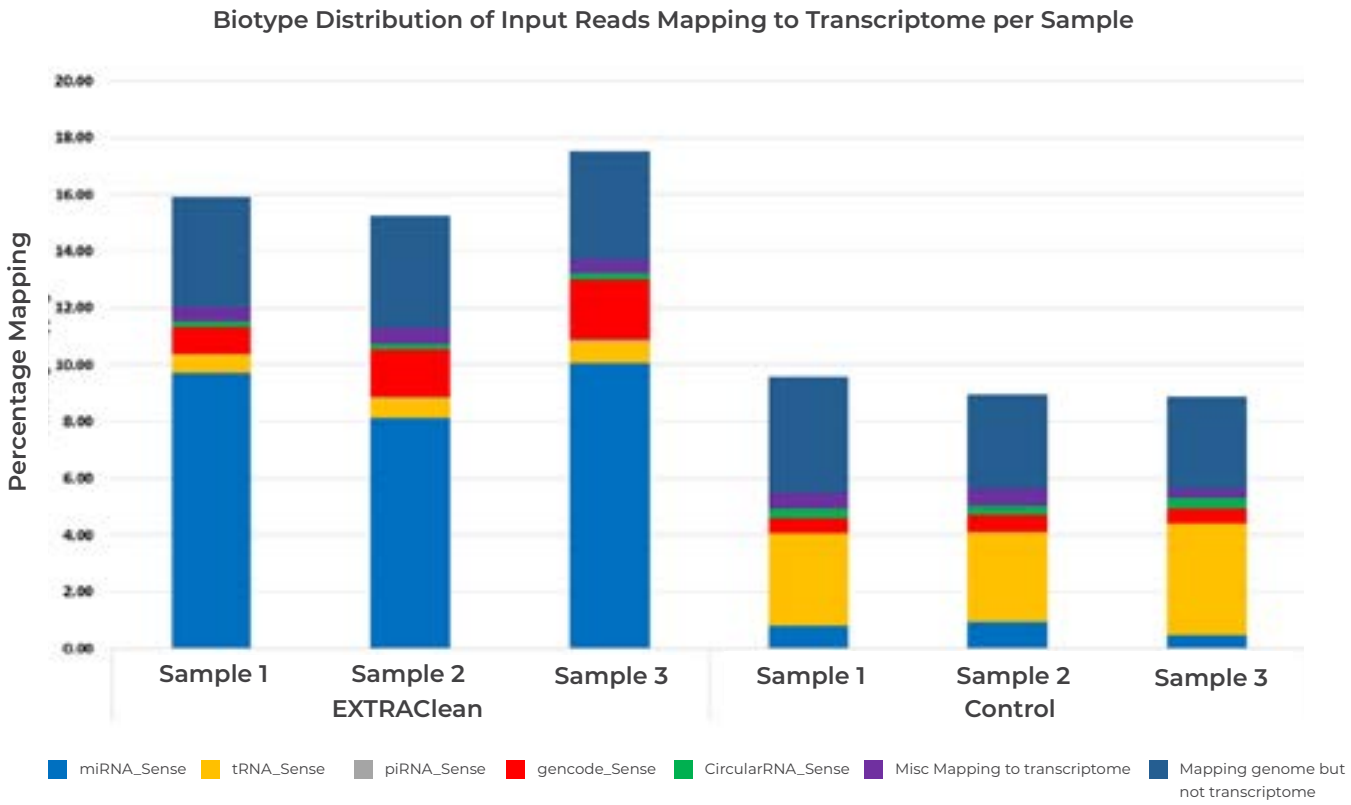

Figure 4. Average small RNA biotype distribution relative to the input reads obtained from sequencing of exosomal RNA extracted from three plasma samples using EXTRAClean Exosomal RNA Isolation Kit (Norgen Biotek Corp., Cat# 72800) and Exosomal RNA Isolation Kit (Norgen Biotek Corp., Cat# 58000). Calculations were relative to raw reads.

Further analysis of the genome mapped reads revealed that the EXTRAClean kit generated approximately 12 times more percentage of miRNA reads as compared to the control kit (EXTRAClean - 1.4 million (9.3%); Control 0.2 million (0.74%) **(Figure 4**). The EXTRAClean kit also generated a higher or similar percentage of other small RNA species as compared to the control kit (EXTRAClean vs Control: piRNA - 0.04% vs 0.01%; circular RNA – 0.22% vs 0.34%). Interestingly, the EXTRAClean kits generated lower tRNA sequences as compared to the control kit (EXTRAClean - 0.7%; Control - 3.45%) **(Figure 4).**

Conclusions and Summary

1. Read size distribution of the EXTRAClean kit is more enriched in miRNA species and is shifted towards smaller RNA sizes.
2. The EXTRAClean kit provides significantly higher percentage of reads corresponding to miRNAs without compromising with other small RNA species such as piRNA and circular RNA.

The EXTRAClean Exosomal RNA Isolation Kit provides high quality RNA that enhances small RNA sequencing outcomes and reduces the fraction of unmapped read fraction. This impacts the NGS economics rendering the run more cost efficient for the obtained value.

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Ordering Info

Description	Prep	Cat. #
EXTRAClean Exosomal RNA Isolation Kit	50 Prep	72800.
Exosomal RNA Isolation Kit	Variable	58000
Small RNA Library Prep Kit for Illumina	24 Prep	63600

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