Comparative Analysis of Plasma and Serum Exosomal Small RNA Sequencing Profiles

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Abstract

Peripheral blood is commonly collected using various devices, with potassium EDTA and serum tubes being prevalent. Plasma and serum serve as primary samples for monitoring disease progression. Exosomes in these samples offer real-time surveillance of prevalent diseases. Extensively studied, exosomal RNA extracted from extracellular vesicles plays a role in regulating transcriptional expression. Due to their low abundance, next-generation sequencing is optimal for characterizing these RNAs. Despite being primary in exosome studies, there's limited data comparing exosomal small RNA sequencing profiles in plasma and serum. This study highlights differences in these profiles from the same donors. Blood was collected from 4 donors in EDTA and Serum tubes. Plasma and Serum were separated and were stored at -80°C until further use. Intact Exosomes were purified from 0.2, 0.5 and 1.0 mL plasma and serum volumes. Extracted exosomes were further processed to extract exosomal RNA. Small RNA library was constructed from all the purified exosomal RNA and sequenced using Illumina's NextSeq 550 platform. A pattern showing an increase in the reads mapped to genome was observed with the increase in plasma (p=0.0122) and serum (p=0.0141) volumes. Similar trend was observed in total small RNA species for plasma (p=0.0037) and serum (p=0.0396) samples. This trend was further reflected in the percentage of reads assigned to miRNA (p=0.0031), piRNA (p=0.042) and rest of the small RNA species combined (gencode; p=0.0473) in plasma samples. Serum samples showed higher percentage of reads mapped to genome as compared to plasma sample, however this was significant only for 1.0 mL serum volume. Among the reads that were mapped to the genome, there was a significant difference in the percentage of miRNA, piRNA and circularRNA reads, between plasma and serum samples. This study reveals a significant impact of sample volume on exosomal small RNA sequencing profiles in plasma and serum. Both plasma and serum exhibited comparable proportions of miRNA, piRNA and circularRNA reads for a 1.0 mL sample volume. At volumes below 1.0 mL (0.2 mL and 0.5 mL), serum demonstrates a higher percentage of small RNA species compared to plasma, suggesting its preference in such cases.

Introduction

Extracellular vesicles (EVs) such as exosomes and microvesicles are gaining importance as their components provide insight into the existing physiological conditions. DNA, RNA, proteins, and lipids that are enclosed in these vesicles carry out important cell-to-cell communications in a healthy or diseased physiological state[1]. To explore the prognostic and diagnostic biomarker capabilities of these EV molecules, it is important to characterize them, as they can potentially be useful in the prognosis or diagnosis of certain pathological conditions including cancer, infections, and autoimmune diseases[1].

RNA profiles are more specific to inter-cellular communications and disease transmission that can be further investigated through sequencing of small RNA[2]. Exosomal miRNA are known to serve as biomarkers for cancers, piRNA for sarcomas, snRNA for pancreatic and colon cancers, circularRNA for gastric tumors, lncRNA for prostate cancer, and snoRNA for lung cancer[2]. These small RNAs, being present in low abundance, makes next-generation sequencing the optimal method for characterizing, quantifying, and analyzing the RNA extracted from EVs obtained from both plasma and serum.

Plasma and serum have undergone thorough examination for exosomal RNA analysis and the sequencing results for each sample type vary due to differences in their RNA profiles. However, the volume of the sample used to purify exosomes might also have an impact on the small RNA sequencing profiles. Hardly any data is available that provides an insight into the minimum amount of starting sample volume that is required to obtain reliable exosomal small RNA sequencing data. In this study, we conducted a comparison of volumes of plasma and serum for the extraction of exosomal RNA to uncover the profiles of small RNA sequencing.

Methods

Blood was collected from four donors to prepare plasma in EDTA tubes (BD, Cat# 366643) and serum in evacuated tubes without any anticoagulant. The separated plasma and serum were further processed to remove cells by centrifuging at 2500 x g for 10 minutes. Cell-free plasma and serum were transferred to a fresh tube and were stored at -80°C until further use. The intact exosomes were purified from 0.2mL, 0.5mL, and 1.0mL plasma and serum volumes using Plasma/Serum Exosome Purification Mini Kit (Norgen Biotek Corp., Cat# 57400). Extracted exosomes were further processed to extract exosomal RNA using the EXTRAClean Exosomal RNA Isolation Kit (Norgen Biotek Corp., Cat# 72800). Extracted RNA was analyzed for quality and quantity on the RNA 6000 Pico assay using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Cat# 5067-1513).

Small RNA libraries were constructed from all the purified exosomal small RNA using the Small RNA Library Prep Kit for Illumina (Norgen Biotek Corp., Cat# 63600). The libraries were quantified on the High Sensitivity DNA Analysis Chip using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Cat# 5067-4626). All libraries were diluted to 4nM concentration, pooled, and sequenced using 51 cycles on the Illumina NextSeq 550 platform using the NextSeq 500/550 High Output Kit v2.5 (75 cycles) (Illumina, Cat# 20024906). Raw reads were analyzed using exceRpt small RNA pipeline[3]. Mapping percentages of quality, genome, and small RNA reads were normalized based on input raw reads, reads used for alignment, and genome-mapped reads, respectively. Correlation analysis was done based on reads normalized by reads per million (RPM) method. Novel miRNA prediction was done using default parameters of miRDeep2 and a cutoff score of 5 was implemented[4]. Simple linear regression analysis and the Welch Two Sample t-test were performed to determine trends and comparisons between different sample volumes.

Results and Discussion

Quality of small RNA sequenced reads improved as the volume of plasma and serum increased (Fig. 1). Although the difference in read quality did not stand out when comparing the serum volumes, the genome mapping distribution showed clear evidence of an increased percentage of human sequences with the increase in plasma or serum volume (Fig. 1 & Fig. 2). An increasing trend in the fraction of genome mapped reads was observed with the increase in plasma (p=0.0122) and serum (p=0.0141) volumes (Fig. 2). A similar trend was observed in the total small RNA species mapping percentage, both for plasma (p=0.0037) and serum (p=0.0396) (Fig. 3).

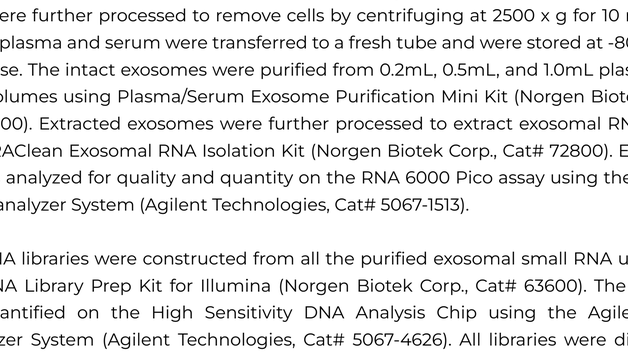

Figure 1. Average read quality distribution relative to raw reads of small RNA sequencing of exosomal RNA extracted from various plasma and serum volumes.

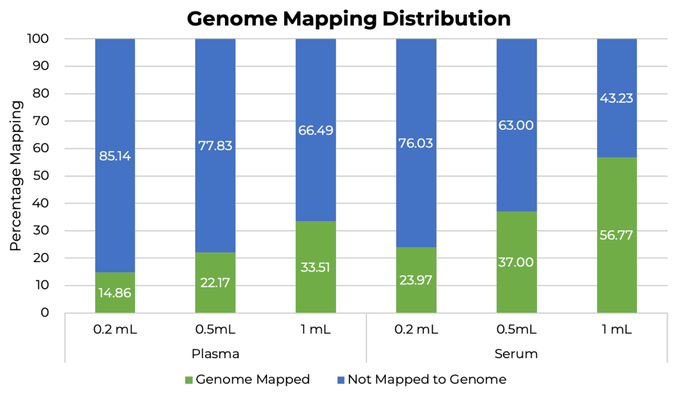

Figure 2. Genome mapping distribution relative to reads used for alignment obtained from small RNA sequencing of exosomal RNA extracted from various plasma and serum volumes.

This trend was further reflected in the miRNA (p=0.0031), piRNA (p=0.042) and gencode (p=0.0473) mapping percentages in the case of plasma samples (Fig. 3). On the other hand, the increase in miRNA and piRNA percentages with the increased serum volumes was non-significant (miRNA: p=0.1577, piRNA: p=0.5754), while an increasing trend in circularRNA mapping percentage was observed in serum samples (p=0.0266) (Fig. 3).

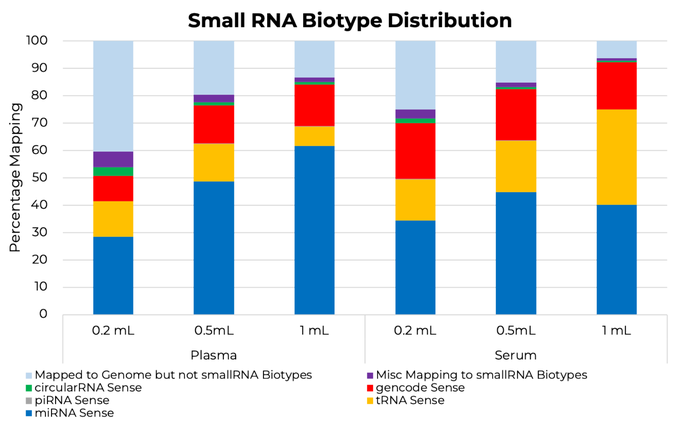

Figure 3. Average small RNA biotype distribution relative to genome mapped reads obtained from sequencing of exosomal RNA extracted from various plasma and serum volumes.

On comparing 0.2mL with 0.5mL and 1mL plasma volumes, the higher volumes (0.2mL vs 0.5mL: p=0.0032; 0.5mL vs 1mL: p=0.0098) exhibited significantly high miRNA mapping (Table 1).

miRNA

Sample type		Serum			Plasma		
Volume (mL)		0.2	0.5	1	0.2	0.5	1
Serum	0.2	1.0000	0.1080	*0.0453*	0.2236	0.2295	*0.0209*
	0.5	0.1080	1.0000	0.3555	0.0621	0.2151	0.8350
	1	*0.0453*	0.3555	1.0000	*0.0344*	0.0794	0.2363
Plasma	0.2	0.2236	0.0621	*0.0344*	1.0000	*0.0032*	*0.0098*
	0.5	0.2295	0.2151	0.0794	*0.0032*	1.0000	0.0600
	1	*0.0209*	0.8350	0.2363	*0.0098*	0.0600	1.0000

piRNA

Sample type		Serum			Plasma		
Volume (mL)		0.2	0.5	1	0.2	0.5	1
Serum	0.2	1.0000	0.1923	*0.0474*	0.3562	0.8616	0.7843
	0.5	0.1923	1.0000	0.6329	0.0692	0.1328	0.1985
	1	*0.0474*	0.6329	1.0000	*0.0064*	*0.0123*	*0.0165*
Plasma	0.2	0.3562	0.0692	*0.0064*	1.0000	*0.0066*	*0.0105*
	0.5	0.8616	0.1328	*0.0123*	*0.0066*	1.0000	0.1996
	1	0.7843	0.1985	*0.0165*	*0.0105*	0.1996	1.0000

tRNA

Sample type		Serum			Plasma		
Volume (mL)		0.2	0.5	1	0.2	0.5	1
Serum	0.2	1.0000	0.2299	*0.0088*	0.0778	0.6561	0.2350
	0.5	0.2299	1.0000	*0.0404*	0.1657	0.2477	0.1901
	1	*0.0088*	*0.0404*	1.0000	*0.0080*	*0.0096*	*0.0086*
Plasma	0.2	0.0778	0.1657	*0.0080*	1.0000	*0.0190*	*0.0408*
	0.5	0.6561	0.2477	*0.0096*	*0.0190*	1.0000	0.0575
	1	0.2350	0.1901	*0.0086*	*0.0408*	0.0575	1.0000

circularRNA

Sample type		Serum			Plasma		
Volume (mL)		0.2	0.5	1	0.2	0.5	1
Serum	0.2	1.0000	0.4685	0.6933	0.1202	0.6202	0.4942
	0.5	0.4685	1.0000	0.5866	*0.0404*	0.3202	0.9945
	1	0.6933	0.5866	1.0000	0.9742	0.8031	0.5882
Plasma	0.2	0.1202	*0.0404*	0.9742	1.0000	0.4561	*0.0494*
	0.5	0.6202	0.3202	0.8031	0.4561	1.0000	0.3324
	1	0.4942	0.9945	0.5882	*0.0494*	0.3324	1.0000

Table 1. Tables showing the statistical significance based on p-value between the different volumes from plasma and serum samples. Analysis was done based on small RNA mapping percentage against genome mapped reads.

Similar observations were made from piRNA (0.2mL vs 0.5mL: p=0.0066; 0.5mL vs 1mL: p=0.0105) and tRNA (0.2mL vs 0.5mL: p=0.0190; 0.5mL vs 1mL: p=0.0408) (Table 1). For circularRNA only 0.2mL and 1mL plasma volume showed a significant difference (p=0.0493) (Table 1). Interestingly, the comparison of 0.5mL with 1mL plasma volumes showed higher miRNA mapping, although non-significant (Fig. 2 & Fig. 3). The number of novel miRNA predicted from 1mL plasma was the highest in comparison to 0.2 and 0.5mL, although non-significant (Table 2).

Volume	Collection tube	Donor number	Number of Novel miRNA	Average	Standard Deviation
0.2 mL	Plasma	D1	28	22.75	9.91
		D2	16		
		D3	13		
		D4	34		
	Serum	D1	20	21.25	5.37
		D2	15		
		D3	22		
		D4	28		
0.5 mL	Plasma	D1	21	20.5	1.73
		D2	22		
		D3	18		
		D4	21		
	Serum	D1	19	25.5	21.97
		D2	15		
		D3	10		
		D4	58		
1 mL	Plasma	D1	30	24.5	5.56
		D2	24		
		D3	17		
		D4	27		
	Serum	D1	21	29.25	6.23
		D2	31		
		D3	36		
		D4	29		

Table 2. Results of Novel miRNA prediction using the miRDeep2 pipeline. Predicted miRNA with score higher than a miRDeep2 score of 5 was used.

Furthermore, the bilateral correlation between miRNA RPM counts of various sample volumes revealed high correlation (R^2> 0.98) between 0.2mL and 0.5mL as well as 0.5mL and 1mL plasma (Fig. 4). A lower correlation (R^2= 0.96) was observed between 0.2mL and 1mL plasma (Fig. 4).

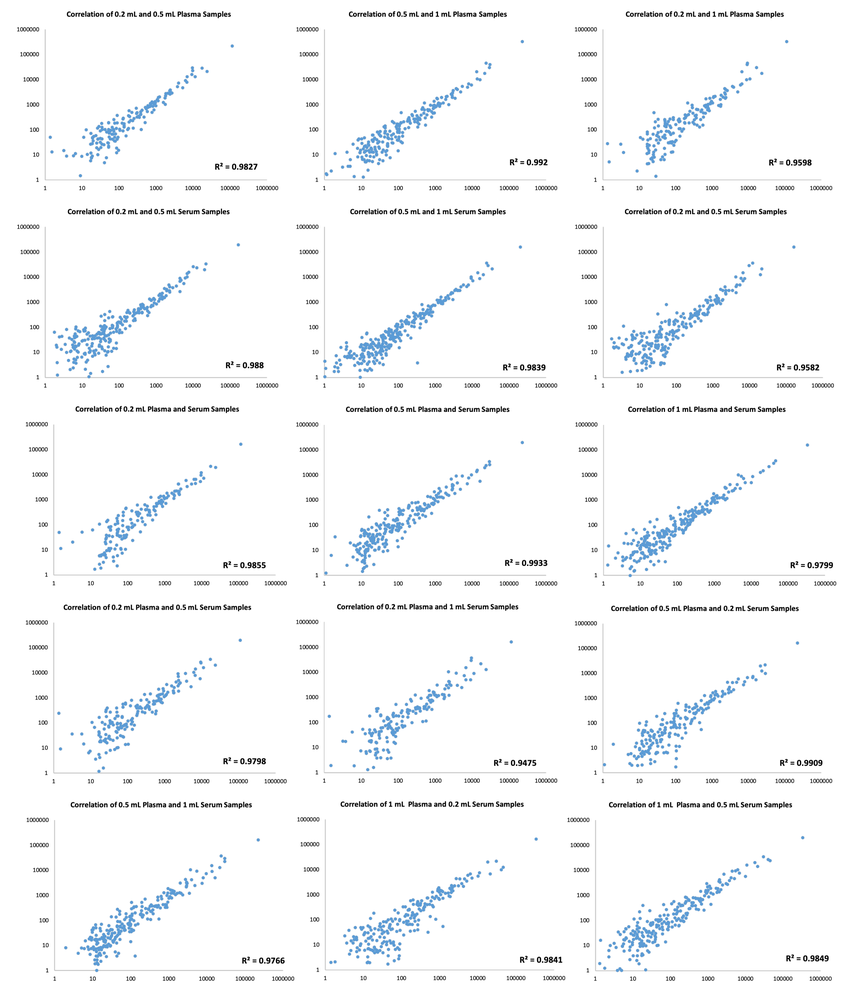

Figure 4. Correlation of miRNA counts normalized at reads per million (RPM) accross different volumes of plasma and serum. Average RPM counts were used per condition to plot the correlation.

Total number of miRNA species detected using 0.5mL and 1mL plasma was significantly higher than 0.2mL plasma, however, the difference in miRNA species between 0.5mL and 1mL plasma had no statistical significance (0.5mL: p=0.0148; 1mL: p=0.0087). Based on the above-mentioned data it is evident that 0.5mL and 1mL plasma gave the best results in terms of miRNA, which is one of the widely studied small RNA species as it has the most diversity and disease association. Based on the various observations, 1mL of plasma demonstrated superior sequencing outcomes in comparison to smaller volumes. Nonetheless, 0.5mL of plasma remains a viable alternative if 1mL is unavailable, and provided that the study does not involve novel miRNA discovery or analysis of low-abundance small RNA.

Based on the difference in the detected miRNA and piRNAs analysis, 0.2mL and 1mL of serum showed significant differences (miRNA: p=0.0453 and piRNA: p=0.0473) but the comparisons at other volumes did not (Table 1). In the case of tRNA significant difference was observed between 0.5mL vs 1mL (p=0.0404) as well as 0.2mL vs 1mL (p=0.0088). Due to the low count of circularRNA the above trends were not observed with detected circularRNA. From the novel miRNA prediction analysis, a tendency of increased miRNAs with the increase of serum volumes was observed (Table 2). Similar to plasma, the bilateral correlation between miRNA RPM counts of various serum volumes revealed high correlations (R^2> 0.98) between 0.2mL and 0.5mL as well as 0.5mL and 1mL of serum, and a lower correlation (R^2= 0.96) between 0.2mL and 1mL of serum (Fig. 4). Regarding miRNA species, only 1mL serum showed significantly higher miRNA counts as compared to 0.2mL (p=0.0308), showing similar miRNA profile between 0.5mL and 1mL serum (p=0.5916) (Fig. 4). The above-mentioned results indicated that 0.5mL serum is optimal for exosomal small RNA sequencing. However, 0.2mL serum can be used if novel miRNA prediction or circularRNA analysis is not required.

The comparison of genome and miRNA mapping percentages between plasma and serum samples revealed that 1mL of serum showed superior results as compared to all volumes (Fig. 1 - 3). In the case of detected piRNA and tRNA, the comparison of 1mL serum showed significantly higher percentages as compared to all plasma volumes (0.2mL of plasma vs 1mL of serum: p=0.0063; 0.5mL of plasma vs 1mL of serum: p=0.0123 and 1mL of plasma vs 1mL of serum: p=0.0164). Detected miRNA species in 0.5mL and 1mL serum were significantly higher than 0.2mL plasma (0.5mL of serum vs 0.2 mL of plasma: p=0.0200; 1mL of serum vs 0.2mL of plasma: p=0.0053). While, detected miRNAs species were significantly higher found in 1mL serum compared to 0.2mL, 0.5mL, and 1mL plasma volumes (0.2mL of plasma: p=0.0053; 0.5mL of plasma: p=0.0207; 1mL of plasma: p=0.0503). Finally, the number of novel miRNAs predicted from 0.5mL serum was similar to 1mL plasma (Table 2). These results show that serum at lower volumes perform better or on power with plasma at 1mL.

Conclusion

- One milliliter of plasma showed the best sequencing outcomes. However, 0.5mL plasma can still be a viable substitute if volume is a constraint as it is non-significantly different as compared to 1mL.
- Both 0.5mL and 1mL serum showed comparable sequencing outcomes, however, both volumes were not significantly higher than the 0.2mL serum. Therefore, 0.2mL of serum can be used if sample volume is a constraint.
- At equivalent volumes, serum performs better in comparison to plasma for exosomal small RNA sequencing.
- Both 1mL plasma and 0.5mL serum volumes are optimal to analyze exosomal small RNA sequencing data with statistical confidence.

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