Stability Evaluation of Plasma Exosomal RNA Stored at Ambient Temperature Collected in Norgen, Streck And EDTA Tubes

Alex Chauhan, M.Sc, Ph.D[1], Lucas Nosal, B.Sc[1], Zakia Dahi, M.Sc[1], Enaam Merchant, M.Sc[1], Taha Haj-Ahmad, M.Sc, Ph.D[1], Moemen Abdalla, M.Sc, Ph.D[1,2], Mohamed El-Mogy, M.Sc, Ph.D[1,2], Yousef Haj-Ahmad, Ph.D[1,2]

[1]Norgen Biotek Corp, Thorold, ON, Canada

[2]Department of Biochemistry, Faculty of Science, Alexandria University, Alexandria, Egypt

Application Note 104

Keywords

+ RNA
+ Tubes
+ EDTA
+ Streck
+ Exosomal
+ Plasma
+ Stability
+ Samples
+ Blood
+ Hemolysis

INTRODUCTION

Exploring the contents of extracellular vesicles (EVs) such as exosomes and microvesicles are gaining importance as they represent the prevailing physiological conditions. These vesicles carry information in the form of RNA, DNA, proteins and lipids that play an important role in the cell-to-cell communication during a healthy or a diseased condition[1]. The characterization of these EV components is thus important to explore their potential as prognostic or diagnostic biomarkers for several pathological conditions including cancers, autoimmune and infectious diseases[1].

Exosomal small RNA sequencing gives an even better understanding of the disease conditions as the RNA profiles are more specific to cell-cell communication and disease transmission[2]. Exosomal miRNA are known to serve as biomarkers for cancers; piRNA for sarcomas, snRNA for pancreatic and colon cancers, circular RNA for gastric tumors, lncRNA for prostate cancer and snoRNA for lung cancer[2]. However, with these exosomes being very fragile, the sample needs careful collection and storage procedures in order to obtain information that is reliable and accurate.

Several blood collection devices are available that can stabilize cell-free RNA (cf-RNA) and extracellular vesicles (EVs) in plasma at room temperature. Streck's RNA Complete BCT tubes and **Norgen's cf-DNA/cf-RNA Preservative Tubes (Cat. 63950)** are among such blood collection devices. Amongst the cargo carried in these EVs, RNA is the most unstable component. Although EDTA tubes are considered the gold standard for plasma separation and subsequent downstream applications, blood collected on the EDTA tubes needs to be processed immediately for plasma separation and needs to be stored at -80°C

cf-DNA/cf-RNA Preservative Tubes
(Cat. 63950, Dx63950) For Whole Blood Collection and Preservation of Cell-Free Circulating DNA and RNA

- Preservation and isolation of both cf-DNA and cf-RNA from a single tube
- Fixative-free preservative, no cross-linking of DNA
- Preserve cf-DNA/ct-DNA for 30 days at ambient temperature and for up to 8 days at 37°C
- Preserve cf-RNA for 30 days at ambient temperature
- Preserve Circulating Tumour Cells (CTCs) for 14 days at ambient temperature
- No plasma volume loss after shipping/transportation
- Prevent hemolysis allowing better separation of plasma
- Prevent apoptosis of blood cells and fragmentation of genomic DNA
- Produce high quality/quantity of plasma cf-DNA/ct-DNA/cf-RNA
- Vacuumed to draw 8.4 mL of blood in 10 mL tubes
- The procedure for plasma isolation using this kit is compliant with ISO 20186-3:2019

before its further use. The capabilities of the blood preservative tubes give freedom to store and ship the samples at room temperature. Streck's RNA Complete BCT tubes claim to preserve cf-RNA and exosomes at room temperature for 7 days while **Norgen's cf-DNA/cf-RNA Preservative Tubes (Cat. 63950)** claim to preserve cfRNA and exosomes for 14 days. In the current study, we assessed the performance of EDTA tubes, Streck's RNA Complete BCT tubes and **Norgen's cf-DNA/cf-RNA Preservative Tubes (Cat. 63950)** to stabilize intact exosomes as well as exosomal RNA at room temperature for the number of days that each tube claims.

MATERIALS & METHODS

Blood from five donors was collected on EDTA (BD, Cat. 366643), Streck's RNA Complete BCT tubes (Cat. 230461), and **Norgen's cf-DNA/cf-RNA Preservative Tubes (Cat. 63950)**. Tubes were stored at room temperature for 0, 5, 7 and 14 days. All the tubes were processed as per the manufacturer's instructions and plasma from each tube was stored at -80°C until further use. Before storing the plasma, an aliquot of 10 µL was made from each tube to measure the hemoglobin concentration by measuring its absorbance at 414nm using NanoDrop 2000. All the plasma samples were thawed and the entire volume of the plasma was used to extract exosomes using **Norgen's Plasma/Serum Exosome Purification Kit (Cat. 57500)** and the exosomes were eluted in 400 µL of ExoR Buffer. An aliquot of 50 µL was sent to The Hospital for Sick Children (SickKids) for NanoSight analysis. RNA was extracted using the **Exosomal RNA Purification Kit (Cat. 58000)** and was analyzed using RNA 6000 Pico assay using Agilent 2100 Bioanalyzer System (Agilent Technologies, USA) as well as Qubit microRNA assay kit.

Small RNA libraries were constructed from all the exosomal RNA samples using the **Small RNA Library Prep Kit for Illumina (Cat. 63600)**. 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 one cycles were used for the sequencing run.

RESULTS & DISCUSSION

Haemoglobin estimation revealed that both EDTA (20.97 mmol/L) and Streck's (17.89 mmol/L) blood collection tubes showed hemolysis on day 0 as compared to Norgen's tubes (7.28 mmol/L). Figure 1 shows the degree of hemolysis in each tube type associated with the preservation period. This hemolysis increased in a time dependent manner for EDTA tubes, while Norgen as well as Streck tubes showed consistency in their hemoglobin concentration (Fig. 2). This was corroborated by the Bioanalyzer 2100 RNA 6000 Pico assay as well as the Qubit microRNA data, as the excessive cellular RNA showed interference with the exosomal

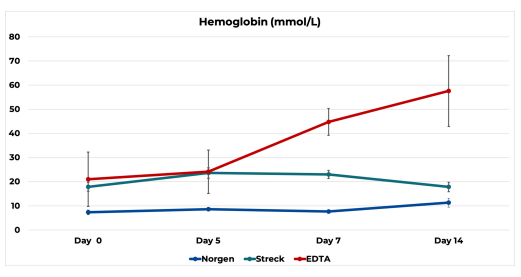

Figure 1. Hemolysis in each tube type across all donors expressed as hemoglobin concentration (mmol/L) as observed on 0, 5, 7 and 14 days post-collection.

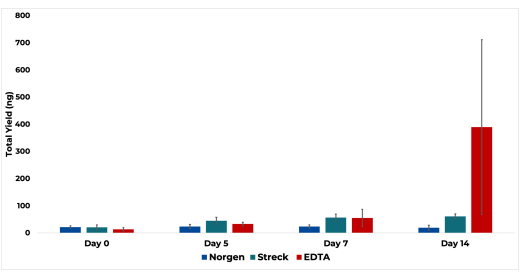

Figure 2. Exosomal RNA yield from day 0 to day 14 for Norgen's cf-DNA/cf-RNA preservative tubes, Streck's RNA Complete BCT tubes and EDTA tubes.

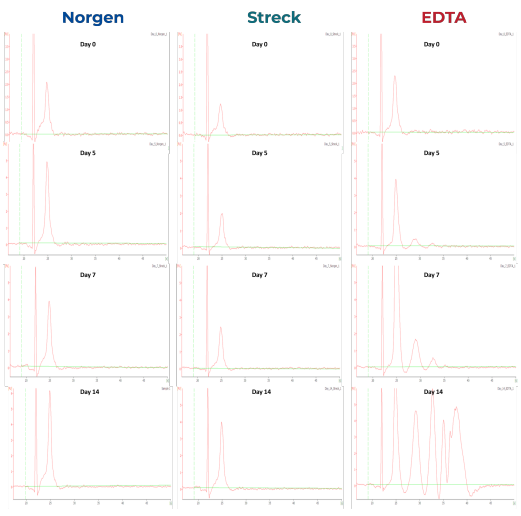

Figure 3. Representative image of quality and quantity analysis of exosomal RNA for each tube type using RNA 6000 pico assay on Agilent Bioanalyzer 2100.

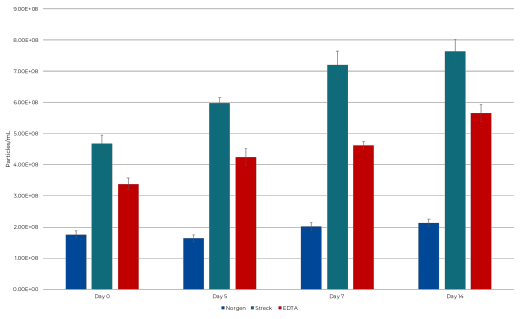

Figure 4. Exosome concentration in Norgen's cf-DNA/cf-RNA preservative tubes, Streck's BCT Complete tubes and EDTA tubes over 14 days post-collection.

enrichment and subsequent RNA extraction (Figs. 3, 4). EDTA tubes showed excessive increase in the RNA concentration while Streck showed a low degree of time dependent increase in RNA concentration. Exosomal RNA extracted from Norgen's tubes consistent RNA yield across all the time points (Figs. 3, 4).

Streck's RNA tubes showed the highest exosome particle concentration (Fig. 5) compared to Norgen and EDTA; which reflects the initial results where Streck and EDTA tubes exhibit hemolysis on Day 0 causing the cellular exosomes to leach into plasma as soon as blood is collected. In addition to exosomal concentrations obtained from NanoSight results, it was evident that the exosomal RNA yield increases exponentially for EDTA (R2=0.997) and Streck (R2=0.8582) tubes, while it was consistent for Norgen's tubes (R2=0.6226) during the course of the 14 day incubation (Fig 6). Moreover, the exosome profiles were not exactly comparable for Streck, Norgen and EDTA, suggesting that a deeper analysis is needed to confirm the purity of non-cellular exosomes from each tube type (Fig. 7).

Cellular exosomes released into plasma due to hemolysis would be purified with cell-free plasma exosomes and lead to inaccurate and unreliable results. Based on hemolysis, Bioanalyzer 2100 data and considering stability claims from each tube type; exosomal RNA extracted from plasma collected on day 0 for EDTA tubes, day 0, 5, and 7 for Streck RNA Complete BCT tubes and day 0, 5, 7, and 14 for **Norgen's cf-DNA/ cf-RNA Preservative Tubes (Cat. 63950)** were further used for small RNA sequencing.

Figure 8 shows consistently low percentage of mapped reads from Norgen tubes compared to that of Streck and EDTA tubes. The increase in miRNA levels observed in both Streck and EDTA tubes is consistent with the concept that the increase in the number of reads is likely to be coming from cellular exosomal RNA that leached due to hemolysis. On the other hand, in contrast to Streck and EDTA, Norgen demonstrated consistent relative reads mapped to miRNA across all the incubation time points for Norgen. Transfer RNA reads had the highest percentage for Norgen (day 0, 5, 7, and 14) as well as Streck (day 0, 5 and 7) (Fig. 8).

The overall distribution of the top 10 miRNA relative to the total miRNA counts across 14 days for Norgen was consistent as it showed abundance of similar miRNA (Fig. 9). However, this consistency could not be observed for Streck as days 5 and 7 showed miRNA abundance that was different from their day 0 (Fig. 9). Further analysis for miRNA abundance revealed that the top 10 miRNA that were common between Norgen, Streck and EDTA tubes were mostly consistent across 14 days for Norgen tubes. On the other hand, Streck tubes showed inconsistencies where some of the miRNA (hsa-let-7f-5p, miR-26a-5p, has-let-7a-5p, hsa-let-7b-5p, miR-146a-5p) showed an increase in reads with increase in storage period (Fig. 10).

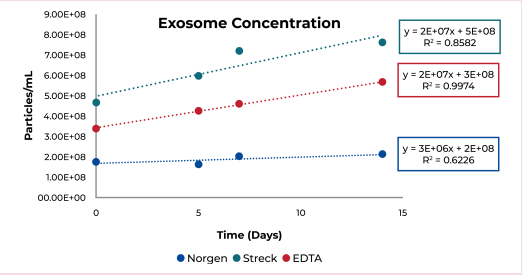

Figure 5. Graph illustrating the concentration of exosomes and the corresponding slope over a period of 14 days for Norgen's cf-DNA/cf-RNA preservative tubes, Streck's BCT Complete tubes and EDTA tubes. The slope and the R2 value for Norgen (R2=0.6226; slope=3.13X106), Streck (R2=0.8582; slope=2.13X107) and EDTA (R2=0.9974; slope=1.63X107) tubes is represented on this graph.

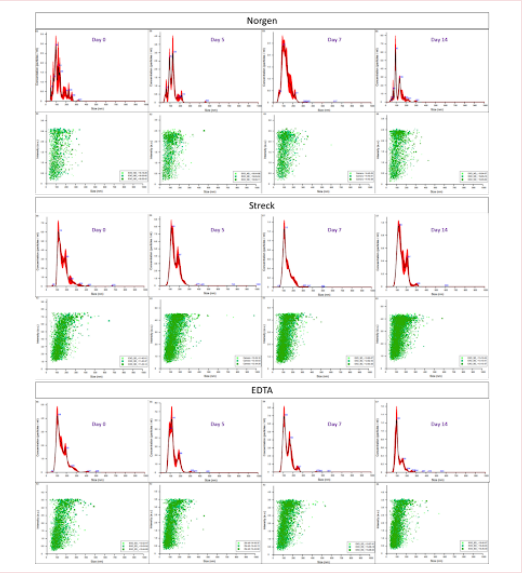

Figure 6. Nanoparticles size distribution analysis: Nanoparticle tracking analysis (NTA) has been performed using NanoSight NS300. Averaged particle size distribution for three measurements indicating a submicron size range between 25 nm and 300 nm with the majority being < 300 nm for all the time points for all the tubes under study.

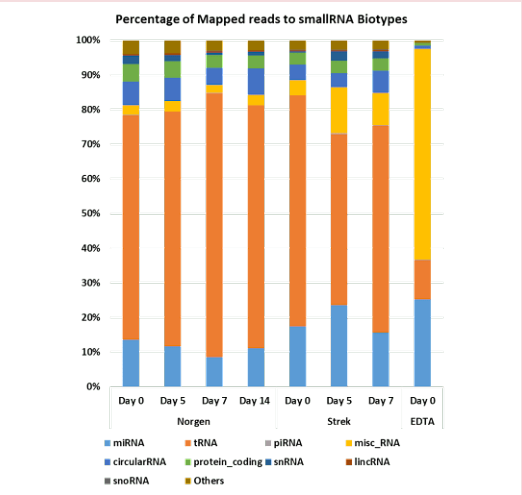

Figure 7. Relative biotype distribution among various blood collection tubes and preservation time.

CONCLUSION

Hemolysis is a key factor in determining the suitable blood collection tube for exosomal analysis. **Norgen's cf-DNA/cf-RNA Preservative Tubes (Cat. 63950)** are associated with lower levels of hemolysis compared to the other tubes, along with higher stability, which results in more reliable and accurate analysis outcomes.

REFERENCES

1. Amorim, M. G., Valieris, R., Drummond, R. D., Pizzi, M. P., Freitas, V. M., Sinigaglia-Coimbra, R., Calin, G. A., Pasqualini, R., Arap, W., Silva, I. T., Dias-Neto, E., & Nunes, D. N. (2017). A total transcriptome profiling method for plasma-derived extracellular vesicles: Applications for liquid biopsies. *Scientific Reports*, 7(1). https://doi.org/10.1038/s41598-017-14264-5

2. Narang, P., Shah, M., & Beljanski, V. (2022). Exosomal RNAS in diagnosis and therapies. *Non-Coding RNA Research*, 7(1), 7–15. https://doi.org/10.1016/j.ncrna.2022.01.001

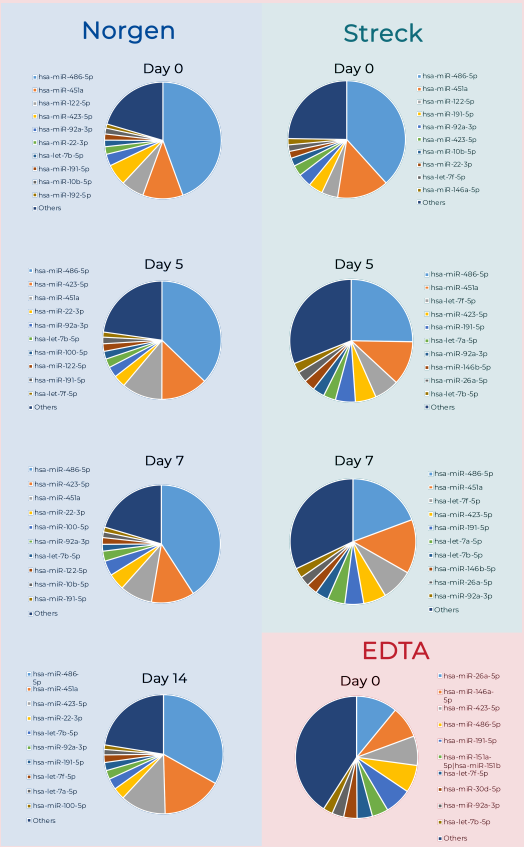

Figure 8. Most abundant (top 10) miRNAs relative to total miRNA counts in each tube type at each time point. Top 10 miRNAs were represented relative to the total miRNA count for for Norgen's tubes (days 0, 5, 7 and 14), Streck's tubes (days 0, 5 and 7) and EDTA tubes (day 0). The rest of the miRNA counts are represented as "others".

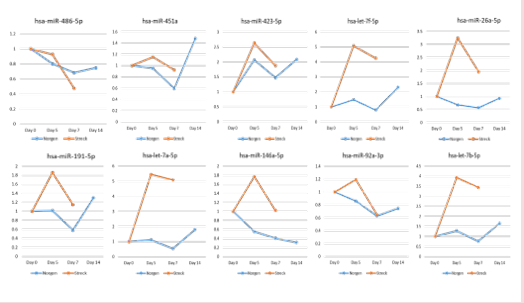

Figure 9. Fold change in common abundant miRNAs of Norgen and Streck tubes. Each miRNA was observed for 14 days in Norgen's tubes and for 7 days in Streck's tubes.

Cat No.	Related Products	Size
63950	cf-DNA/cf-RNA Preservative Tubes	50 Tubes
Dx63950	cf-DNA/cf-RNA Preservative Tubes Dx CE	50 Tubes
57500	Plasma/Serum Exosome Purification Kit	50 Preps
58000	Exosomal RNA Purification Kit	Variable
63600	Small RNA Library Prep Kit for Illumina (Indexes 1-24)	24 Preps

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

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

v2.1 | Revised on July 18th, 2023

For more information about our products, scan the QR Code or visit our website.