Blood Collection Tube Selection and Storage Time Impact the Quantity and Quality of Cell Free Total Nucleic Acids

Hannah Saunders, Natalie Supunpong Hernandez , Jennifer Whiting, Charmaine San Jose Hinahon, and Angie Cheng

Thermo Fisher Scientific, 2130 Woodward St, Austin, TX 78744

ABSTRACT

Liquid biopsy is emerging as a non-invasive companion to traditional solid tumor biopsies. There are a number of blood collection tubes (BCTs) targeted to the liquid biopsy market offering stabilization of circulating cell-free nucleic acid (cfNA = cfDNA and cfRNA) profiles. Liquid biopsy samples are often limited, and may be subjected to handling or storage conditions that contribute to unwanted genomic DNA (gDNA). Low cfNA yields can limit the amount of the target available for next generation sequencing (NGS) liquid biopsy assays, possibly impacting sequencing results. It is important to understand the impact of BCTs and storage time on quantity and quality of cfNA. We explore the impact of select BCTs on cfNA and discuss considerations for selecting a tube optimally suited to liquid biopsy studies.

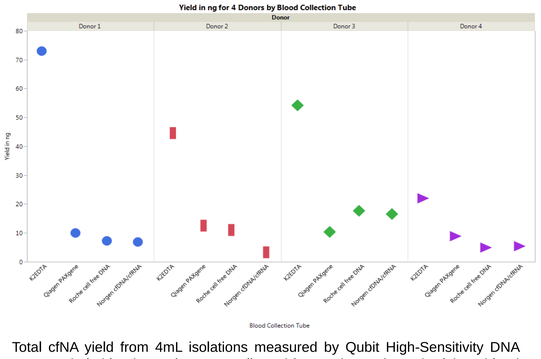
MATERIALS AND METHODS

In the first part of the study, we selected a variety of tubes to survey for performance 24 hours post blood draw. Two tubes were collected from each of the 4 tubes types for all donors to allow for comparisons among the blood collection tubes. Whole blood was collected in BD Vacutainer® K2EDTA, Roche Cell-Free DNA Collection Tube, Norgen Biotek cf-DNA/cf-RNA Preservative Tubes, and Qiagen PAXgene™ ccfDNA Tubes and stored at room temperature for 24hrs. The cfTNA was isolated from 4 mL of cell free plasma (duplicate is isolations) for each tube using the Kingfisher™ Flex Magnetic Particle Handler and the MagMAX™ Cell-free Total Nucleic Acid Isolation Kit. Samples were assessed using Qubit™ High Sensitivity DNA Assay, Agilent 2100 Bioanalyzer™ with a High Sensitivity DNA Kit and RNA was assessed via RT-qPCR with m1 assays. Based on the date from this study, a follow up study was designed to examine the BD Vacutainer® K2EDTA, Streck cfDNA, and Qiagen PAXgene tubes more closely.

Next, we examined K2EDTA Streck cfDNA, Qiagen and PAXGene tubes with whole blood held at room temperature for 1,2,3,10, and 30 days prior to cell free total nucleic acid isolation. To collect enough material for all time points, different donors were used for each blood collection tube. We examined how the yield and profile of the cell free nucleic acids changed over time when blood was stored in the collection tube. Samples were isolated using the Kingfisher™ Flex Magnetic Particle Handler and the MagMAX™ Cell-free Total Nucleic Acid Isolation Kit. Libraries were prepared for the Ion Torrent™ Oncomine Lung Cell-Free Total Nucleic Acid Research Assay Sequencing libraries using maximum volume input for all samples (10.4 µl), and subsequently mass normalized when pooling for NGS. Templating and sequencing occurred on the Ion Chef™ & Ion GeneStudio S5™ System. Analysis was performed with Ion Reporter™ v5.6 software using the Oncomine™ TagSeq Lung Liquid Biopsy workflow

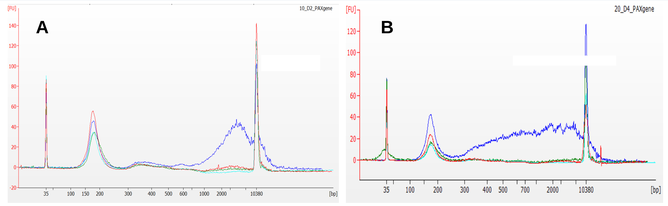
RESULTS

Figure 1 – Total yield by Blood Collection Tube for 4 Donors



Total cfNA yield from 4mL isolations measured by Qubit High-Sensitivity DNA Assay. Whole blood samples were collected from 4 donors in each of the 5 blood collection tubes, shipped overnight and cfTNA isolated 24hours after blood collection. K2EDTA had the highest yields for all 4 donors, however, these higher Qubit yields reflect the increased amount of gDNA present in the K2EDTA tubes (see Figure 2) Yields for the stabilizing tubes were lower but lacked gDNA contamination. Yield from the Qiagen PAXgene blood collection tube was the highest among the stabilizing tubes tested at this 24 hour time point overall.

Figure 2 – Nucleic acid size profiles from blood collection tubes at 24 hours

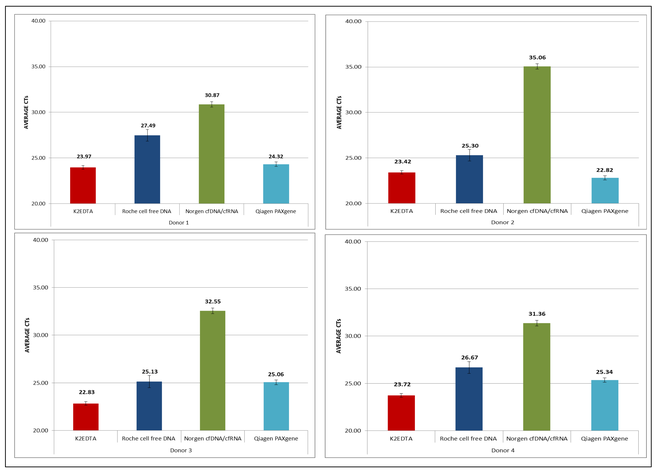


Blue=K2EDTA Green=Roche Teal=Norgen Red: Qiagen

Nucleic acid size profiles were analyzed on the Agilent 2100 Bioanalyzer using the High Sensitivity DNA Kit. Representative traces from 2 of the 4 donors tested are shown above. Results from the other 2 donors were comparable, with the K2EDTA samples yielding even higher gDNA amounts than those above, aligning with Qubit results from Figure 1. Detection of genomic DNA (gDNA) in the sample is an important quality control metric, as gDNA may interfere with library amplification.

Figure 3 – RNA by RT qPCR and NGS Control Gene Family Counts

A



B

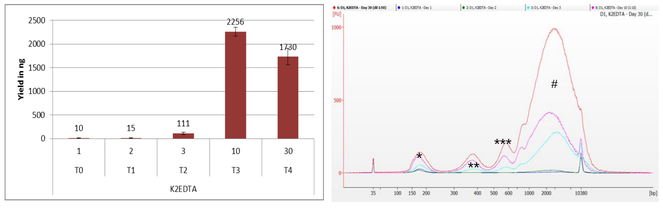
Blood Collection Tube	Mass Input (ng)	TBP Families per ng library input	HMBS Families per ng library input
K2EDTA	20	7.8	5.6
Roche cell free DNA	9.2	4.7	14.1
Norgen cfDNA/cfRNA	8.6	0.23	0.12
Qiagen PAXgene	5.4	20.4	32.4

A) RTqPCR using two m1 targets (detects RNA only) was performed to assess RNA across various tube types. Two targets, ACTB (171bp) and GAPDH (122bp), revealed a similar pattern with K2EDTA generally showing the lowest Cts followed by Qiagen PAXgene ccfDNA Tubes. ACTB results are shown for all 4 donors and all 4 tubes.

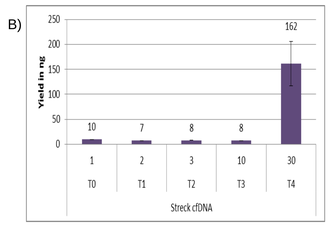
B) Donor 3 was selected for NGS using the Oncomine Lung Cell-Free Total Nucleic Acid Research Assay. The panel contains two low-to-moderately expressed RNA targets that act as control genes. Input volume was maximized at 10.4uL. Coverage and mean read length were comparable between all tube types for Donor 3. Per ng of cfTNA used for library input, PaxGene tubes have the most RNA families for HMBS and TBP RNA controls.

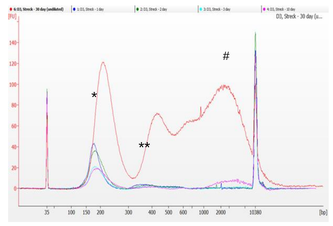
Figure 4 – Total yield and profile over time by Blood Collection Tube

A)

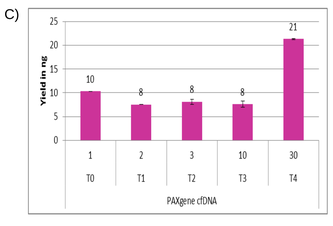


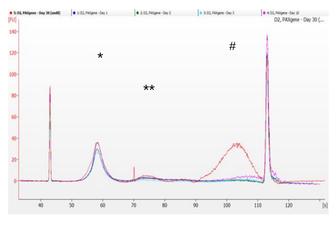
B)





C)





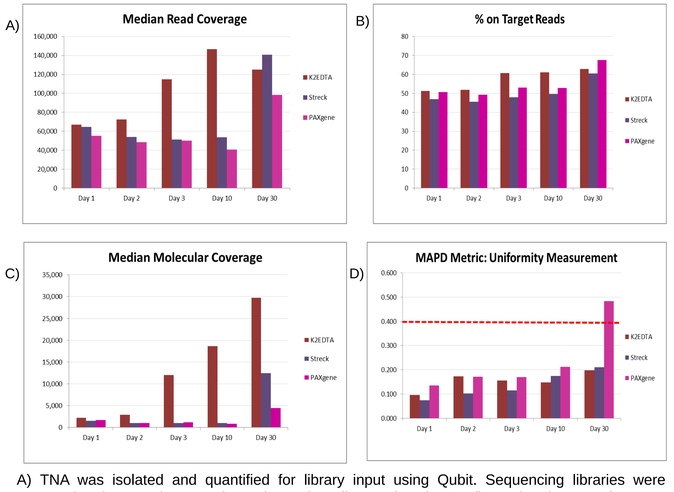
A) In whole blood stored in K2EDTA tubes, gDNA levels began increasing at 3 days. The cell free fraction appears to increase as well but is likely due to fragmentation as the gDNA degrades.

B) In whole blood stored in Streck cfDNA tubes, gDNA does not increase dramatically until the 30 days time point. For these tubes it is important to note the cell free peak is the highest at day 1 and 2 likely reflecting the continued fixation of the cell free nucleic acid over time thus making it more difficult to free.

C) In whole blood stored in PAXgene ccfDNA, gDNa levels did not increase until the 30 day time point. The cell free DNA yields remained consistent at the earlier time points.

(*) Primary cfDNA peak, (**), di-nucleosomal cfDNA, (***) tri-nucleosomal cfDNA, (#) high molecular weight gDNA contamination. Comparisons cannot be made across tube types in the se experiments as different donors were used for each tube type.

Figure 5 – Impact of blood collection tube and size profile over time on NGS metrics



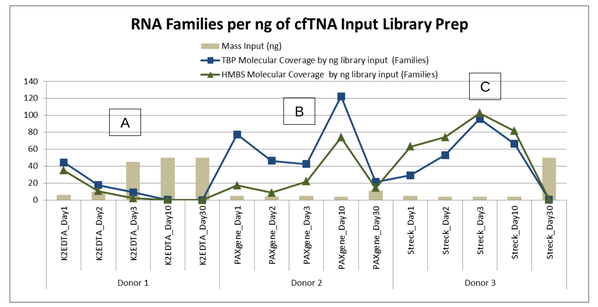
A) TNA was isolated and quantified for library input using Qubit. Sequencing libraries were prepared using maximum volume input for all samples (10.4 µl), and subsequently mass normalized when pooling for NGS. Samples were templated using the Ion Chef™ (four 530 chips, pooled by tube type), sequenced on the GeneStudio S5™ System and uploaded to Ion Reporter for analysis. Mean Read Coverage for all samples on all chips was at or over 40,000.

B) Percentage of On Target Reads for all tubes and chips. A small increase can be observed at day 3 with another increase at day 30.

C) Median Molecular Coverage increased as the proportion of the gDNA to cfDNA in the sample increased. Preservative containing tubes held steady until between days 10 and 30.

D)The median absolute pairwise difference (MAPD) quality control metric was higher exceeded the ion Reporter threshold of <0.4 on the day 30 PAXGene sample. The MAPD metric is important for CNV detection, and therefore accurate CNV calling may be negatively impacted in this sample.

Figure 6 – RNA Family Counts for Control Genes TBP and HMBS



NGS RNA family counts for 2 control genes are shown for each time point to examine RNA preservation over time. Family counts for both genes are overlaid with the TNA mass input in ng used for the Oncomine Lung Cell-Free Total Nucleic Acid Research Assay.

A) K2EDTA tubes had the highest counts on day 1 confirming the need to process blood from these tubes as quickly as possible.

B) PAXgene counts were steady for HMBS for days 1, 2, and 3. The TBP counts dropped from day 1 to day 2. Both targets spiked at Day 10 and dropped at day 30.

C) Streck counts increased gradually for both targets for days 1, 2 and 3 and then dropped off at days 10 and 30.

CONCLUSIONS

- K2EDTA tubes had the highest total yield by Qubit (yield/mL of input ranged from 2.32-7.45ng), but also had the most gDNA contamination by Bioanalyzer.
- Stabilizing BCTs had lower yields than K2EDTA, with a reduction in both cfNA and gDNA. However, gDNA was greatly reduced in all stabilizing BCTs compared to matched K2EDTA. cfDNA decreased by varying amounts with no clear standout in maintaining the cf fraction over time
- Roche and Norgen had higher cfRNA Ct values (2-3 Cts for Roche or 6-10 Cts for Norgen) than K2EDTA and PAXgene when the same donors were used to compare differences among all tube types.
- In the time course experiment, cfDNA decreased over time by Bioanalyzer profile. K2EDTA yields began increasing by 48hrs due to an increase in gDNA. Yields were steady for the remaining tubes until day 10, when gDNA contamination increased.
- Blood collection tubes may impact RNA stability and subsequently recovery. Blood collection tube selection and storage time may impact NGS metrics for RNA targets.

Blood collection tubes can impact the quantity and profile of nucleic acid required for downstream assays. Maximum yield of the cell free fraction, minimal gDNA contamination must be balanced with stability and convenience from collection to processing to achieve optimal downstream sensitivity and specificity.
If blood cannot be processed within 24 hours, a stabilizing BCT may be used to extend storage at room temperature for up to 48 hr with minimal impact.
If RNA targets are critical to downstream applications, a stabilizing blood collection tube may be a desirable first choice as RNA likely begins to degrade quickly post blood collection.

REFERENCES

1. Ward Gahlawat, A.; Lenhardt, J.; Witte, T.; Keitel, D.; Kaufhold, A.; Maass, K.K.; Pajtler, K.W.; Sohn, C.; Schott, S. Evaluation of Storage Tubes for Combined Analysis of Circulating Nucleic Acids in Liquid Biopsies. Int. J. Mol. Sci. 2019, 20, 704.

2. Page K, Guttery DS, Zahra N, Primrose L, Elshaw SR, Pringle JH, et al. (2013) Influence of Plasma Processing on Recovery and Analysis of Circulating Nucleic Acids. PLoS ONE 8(10): e77963. https://doi.org/10.1371/journal.pone.0077963

TRADEMARKS/LICENSING