Creation of contrived plasma samples with RNA variants to use as Liquid Biopsy reference material.

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INTRODUCTION

Liquid biopsy using circulating cell free nucleic acid is revolutionizing oncology due to its non-invasive nature. However, challenges like the limited amount of blood that can be drawn from a human donor and the extremely low abundance of tumor derived DNA/RNA molecules in the blood results in insufficient amounts of cell free total nucleic acid needed for liquid biopsy studies. These challenges prompted the need for contrived plasma samples to attain enough cell free total nucleic acid for conducting liquid biopsy studies.

RNAs are unstable molecules that are prone to degradation by nucleases. Therefore, circulating cell-free RNAs (Cf RNA) in blood are encapsulated within extracellular membrane vesicles, or they form ribonucleotide complexes that protect them from nuclease activity (Tzimagiorgis *et al.,* 2011). In addition, the presence of high concentration of RNases (ribonucleases) in blood degrade exogenous RNA as a part of natural defense system from infectious nucleic acids, which poses the most challenging aspect for creation of contrived plasma samples. Current study explores different methods for creating contrived plasma samples containing stable RNA variants, which were tested downstream by next generation sequencing to detect the variants.

METHODS

Different methods were explored to stabilize RNA variant containing plasma to create contrived plasma samples that would mimic the real variant positive samples. Out of the different methods tested, three methods (proprietary) for introducing the RNA variants into healthy donor plasma showed promising results. The workflow for testing the contrived plasma samples is given in figure 1 below. To evaluate these methods, following the introduction of Cell free Total Nucleic Acid (Cf TNA) containing RNA variants into a plasma pool, nucleic acid was isolated using a Genexus™ Cell-Free Total Nucleic Acid Purification Kit and Genexus™ Purification System. The isolated nucleic acid was sequenced on Ion Torrent™ sequencing platform (reference provided) using AmpliSeq™ HD target amplification assay to detect the presence of variants that were spiked in. This assay measures both DNA and RNA variants simultaneously from CfTNA material. Number of detected molecules containing the RNA variant in Cf TNA is specified by molecular counts in this assay.

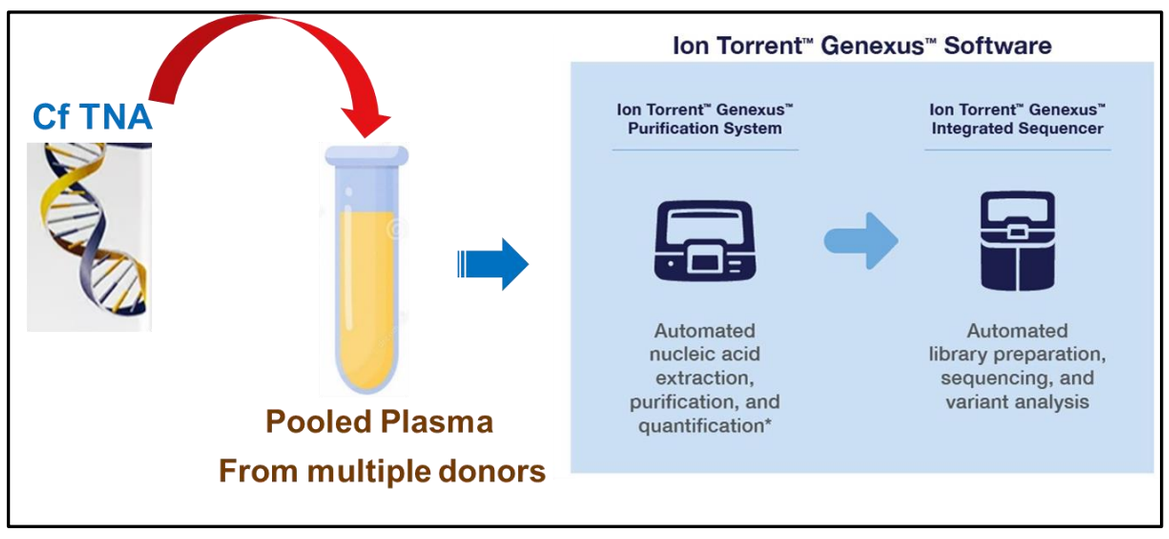

Figure 1. Workflow for isolation of cell free total nucleic acid from contrived plasma samples using Genexus purification instrument and sequencing the nucleic acid downstream on Genexus sequencer to detect the fusion variants. Pooled plasma is used for creation of contrived samples to account for donor variability. Cf TNA used for spike in to create contrived plasma was isolated from cancer cell media positive for RNA variants.

RESULTS

Different options were explored to create contrived plasma samples to mimic real variant positive samples and feasibility data showed successful creation of contrived plasma samples with stable RNA variants for detection. Two of the methods (proprietary methods 1 & 2) tested showed that exogenous nucleic acid containing RNA variants spiked into plasma is stabilized by the methods used and showed increased recovery rates as shown in figure 2. Recovery rates varied depending on the variant levels in stock Cf TNA's being used for spike in.

To understand the dose response of these spiked in RNA variants in plasma by the proprietary methods used, different ratios of variant and healthy donor plasma were mixed (method-3) and tested for variant detection (figure 3). Results from this experiment shows a linear recovery trend indicating a good correlation between input amount of the RNA variant used and recovery of that variant. Data for this experiment is shown in figure 3. In addition, to test overall stability of the RNA variants spiked into plasma by the proprietary methods used, freeze thaw events were performed on these contrived plasma samples and sequencing was performed on Cf TNA isolated from them (figure 4).

Successful creation of contrived plasma samples with stabilized RNA variants to go through the workflow to measure the variants that were spiked in

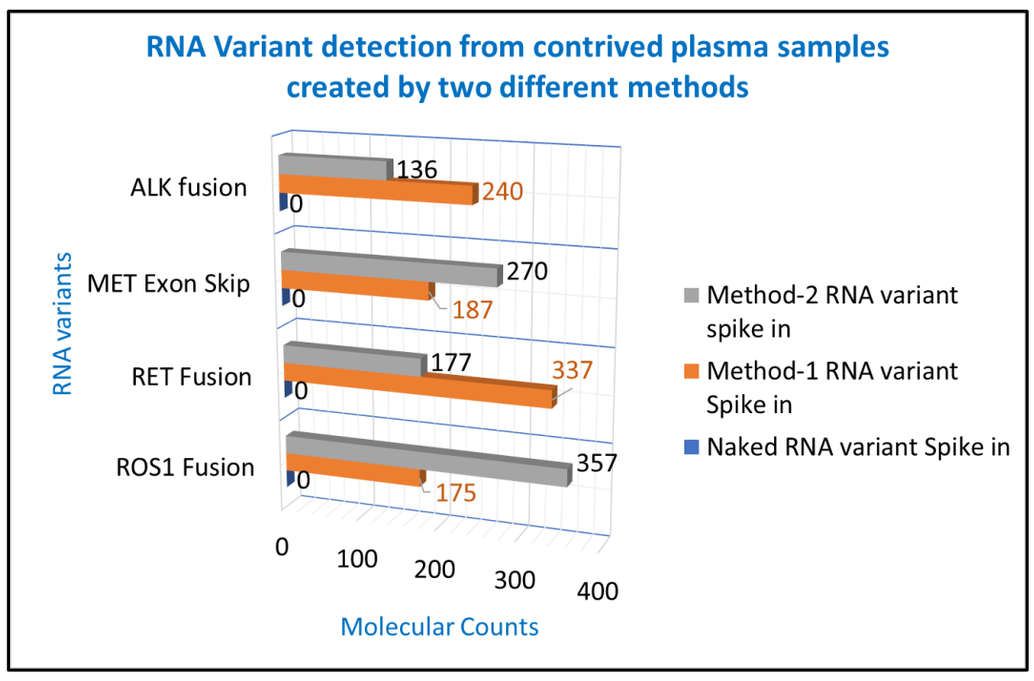

Figure 2: AmpliSeq™ HD target amplification assay data showing the presence of four RNA variants from the contrived plasma samples created by two different methods (1 & 2). The fusion calls for four RNA variants is shown by molecular counts. Contrived plasma with naked RNA variants is shown for comparison to show the efficiency of method-1&2 in recovering RNA variants.

Dose response of the RNA variant in contrived plasma samples

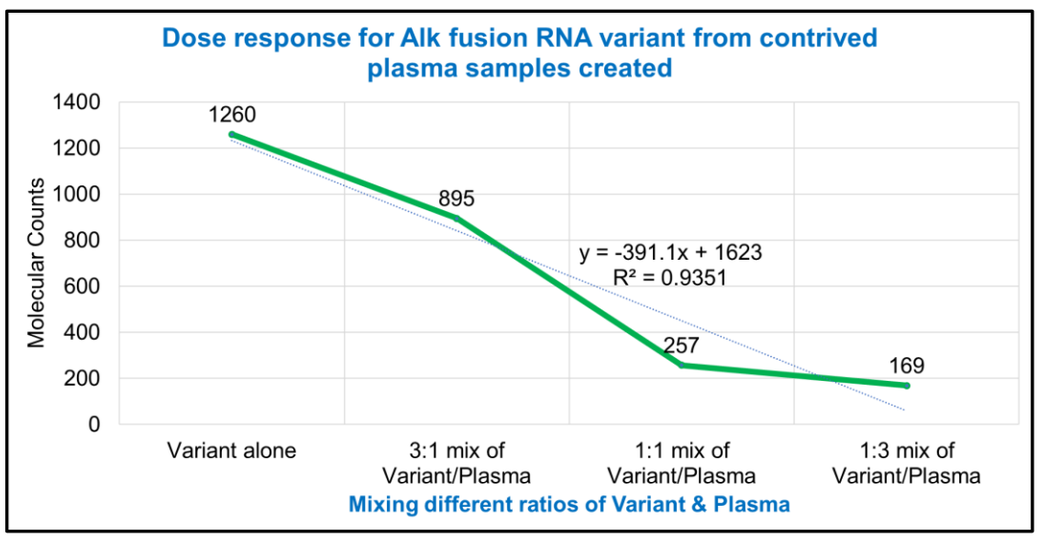

Figure 3: AmpliSeq™ HD target amplification assay data showing the detection of RNA variants from the contrived plasma samples created by mixing different ratios of variant with plasma (method-3). The fusion calls for ALK fusion variant is shown by molecular counts in the graph & good linear correlation of input amount and recovery is observed.

Stability of an RNA variant after multiple freeze thaws performed on contrived plasma samples

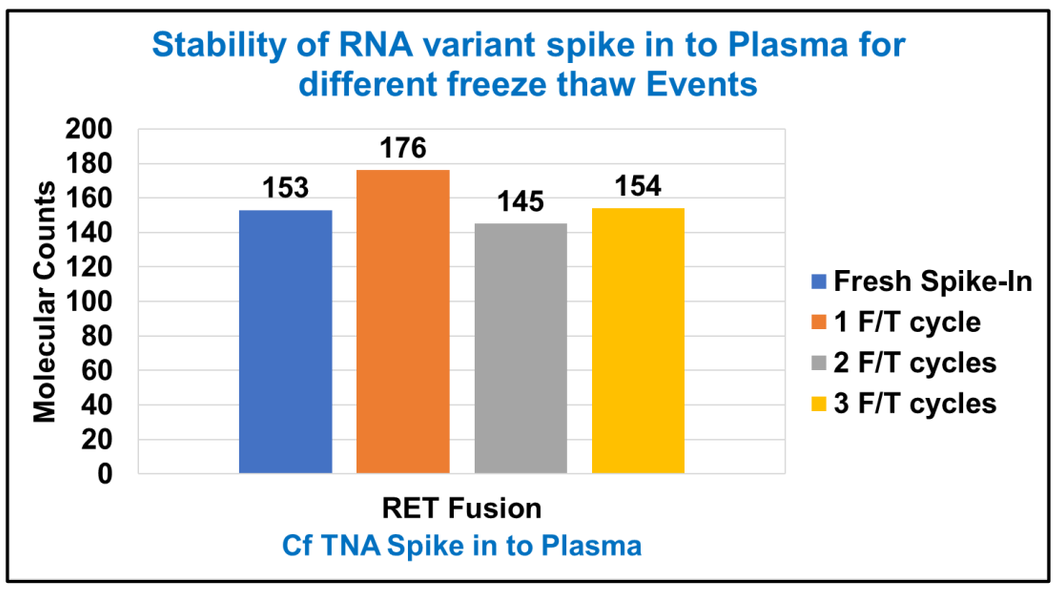

Figure 4: Sequencing data showing the stability of RET fusion variant after multiple freeze thaw events performed on contrived plasma samples created by method-2. Fresh spike in of RNA variant into plasma is shown for comparison.

CONCLUSIONS

This study demonstrates the successful creation of contrived plasma samples with RNA variants that mimic the biological complexity of human blood samples, which can be used in liquid biopsy workflows. The use of both surrogate and contrived samples can foster innovation, when real variant positive samples are difficult to obtain.

REFERENCES

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