Cell-free RNA next-generation sequencing workflow for the Streck RNA Complete BCT®

Next-generation sequencing has revolutionized medicine. RNA-based whole-transcriptome approaches provide a valuable dynamic view of disease. However, a hurdle in said testing is that patient samples are not stable and RNA-sequencing experiments target low levels of disease-specific cell-free RNA. This workflow describes the steps Streck used to perform cell-free RNA-sequencing.

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Introduction

Next-generation sequencing (NGS) has revolutionized medicine with current applications stimulating the rapid growth of targeted treatment strategies.[1] This is most apparent in the setting of oncology. While DNA-based whole-genome or whole-exome sequencing has produced valuable information related to genomic complexity (point mutations, insertions, deletions, etc.), it is limited to a static footprint of the disease state. RNA-based whole-transcriptome approaches, such as RNA-seq, provide a more dynamic view of the disease.

Specifically, RNA-seq methods are used to determine which genes or regulatory pathways are expressed at any given point in time or under a certain therapeutic condition.[2,3] Results are directly actionable and can be used to guide personalized treatment strategies. While traditional patient biopsies work for this application, they are invasive and generally biased by the site of excision. Liquid biopsies, on the other hand, are noninvasive homogenous samples that better reflect the global disease state.[4] Importantly, liquid biopsies can be repetitively and easily obtained, thus opening the possibility of straightforward disease monitoring.

Background

A hurdle in making liquid biopsy commonplace in the clinic is the fact that patient samples are not stable. Whole blood samples drawn into standard tubes (e.g., EDTA) will degrade over time, including the white blood cells, erythrocytes, and platelets.[5] One such analyte that is exceptionally sensitive to storage time-dependent cellular breakdown is cell-free RNA. Cell-free RNA (cfRNA) molecules, including mRNAs, miRNAs, and lncRNAs, are generally assumed to be encapsulated within extracellular vesicles (EVs), such as exosomes.

Cellular deterioration in unstabilized patient samples leads to increases in disease non-specific EVs and EV-associated cfRNAs which in turn dilute out the concentration of disease-specific cfRNA in the analyte pool.[6,7,8] This becomes problematic for RNA-seq experiments aimed at detecting disease-specific cfRNAs which typically present at very low concentrations. **The Streck RNA Complete BCT® maintains draw time concentrations of EVs, and thus cfRNA, by preventing blood cell breakdown during the storage and/or shipment process.**

Materials

1. Blood from self-declared healthy individuals collected into Streck RNA Complete BCTs.
2. Cell-free RNA isolation kit, such as QIAamp Circulating Nucleic Acid Kit from Qiagen (Cat# 55114)
3. DNase Digestion kit, such as the RNase-Free DNase Set from Qiagen (Cat# 79254)
4. RNA cleanup kit, such as RNeasy MinElute Cleanup Kit from Qiagen (Cat# 74204)
5. Agilent RNA 6000 Pico Kit (Cat# 5067-1513)
6. RNA quantification kit, such as Qubit RNA HS Assay Kit (Cat# Q32852)
7. NEBNext rRNA Depletion Kit from New England BioLabs (Cat# E6310)
8. NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (Cat# E7760)
9. NEBNext Multiplex Oligos for Illumina (Cat# E7335, E7500, E7710, E7730, E6609, or E7600)
10. NEBNext Library Quant Kit for Illumina (Cat# E7630)
11. Agilent High Sensitivity DNA Kit (Cat# 5067-4626)
12. User defined Illumina sequencing kit (e.g., NextSeq 500/550 High Output Kit v2.5)

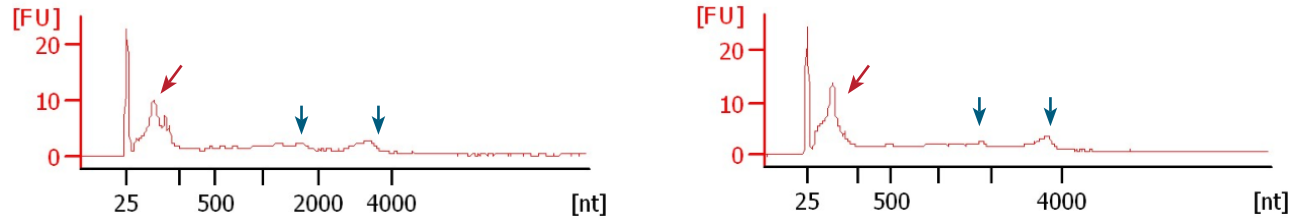
Cell-Free RNA Isolation

This step utilizes the QIAamp Circulating Nucleic Acid Isolation Kit as an example. Others that 1) do not utilize Phenol-Chloroform and 2)

incorporate a Proteinase K step are applicable with examples including the Plasma/Serum Circulating and Exosomal RNA Purification Kit from Norgen Biotek and the MagMAX Cell-Free Total Nucleic Acid Isolation Kit from Thermo-Fisher Scientific.

1. Isolate plasma from whole blood samples using a double spin protocol consisting of a primary spin at 1800 g for 15 minutes followed by a secondary clarification spin at 2800 g for 15 minutes. Transfer 3 mL of plasma to 50 mL conical tubes and proceed directly to cfRNA isolation. Alternatively, plasma samples may be stored at -80 °C until processing.
   a. RNA Complete BCTs may be processed up to seven days post blood collection when stored at room temperature.
   b. The double spin protocol described herein was shown by Streck R&D to deplete all detectable platelets as assayed by a commercial hematology instrument.
2. Purify total nucleic acid with the QIAamp Circulating Nucleic Acid Kit using the manufacturer provided “microRNA protocol” for 3 mL of plasma. The following modifications are added to the protocol.
   a. Extend the 60 °C incubation from 30 minutes to 60 minutes.
   b. Elute with 87.5 µL of Buffer AVE.
3. Immediately perform a DNase I digestion of the eluted nucleic acids according to the manufacturer’s recommendation for “DNase Digestion of RNA before RNA Cleanup” in the QIAmp Circulating Nucleic Acid Kit handbook.
4. Immediately perform clean-up of the cfRNA using the RNeasy MinElute Cleanup Kit according to the manufacturer’s recommendations for “Clean-Up of RNA solutions after DNase Treatment” as outlined in the QIAmp Circulating Nucleic Acid Isolation Kit handbook.
   a. Elute with 18 µL of RNase-free water.
   b. Keep the eluted RNA on ice for short-term use or immediately freeze at -80 °C for long-term storage.
5. Perform quantitative analysis of cfRNA using the Qubit RNA HS Assay Kit and a quality check using the Agilent Bioanalyzer with the RNA Pico 600 kit (both according to manufacturer recommendations). Purified samples are typically highly fragmented (RIN score of 2-4) and concentrations are in the low nanogram per µL range (2-20ng/mL plasma). See **Figure 1** for representative Bioanalyzer traces from both EDTA and RNA Complete BCT samples.

Figure 1: Qualitative analysis of representative cfRNA isolations



EDTA Tubes | **Streck RNA Complete BCT®**

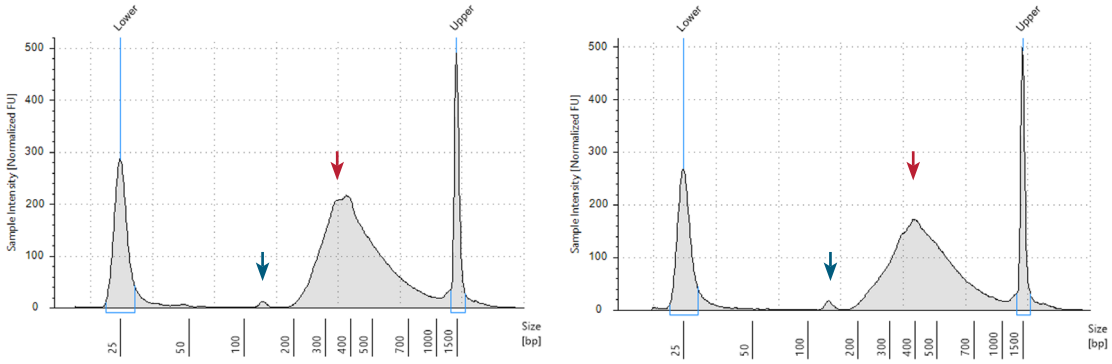
Blood samples were drawn into EDTA (legacy) tubes or Streck RNA Complete BCT, mixed following IFU, and plasma isolated. Total nucleic acid was purified, DNase-digested, concentrated, and then run on the Agilent Bioanalyzer using the RNA 6000 Pico Kit. The blue arrows indicate larger 18S and 28S ribosomal RNA peaks while the red arrows denote smaller and highly fragmented RNA species.

Library Preparation

1. Using 12 µL of the purified cfRNA, complete ribosomal RNA (rRNA) depletion using the NEBNext rRNA Depletion Kit according to the manufacturer’s recommendations.
   a. Critical points for this step include use of a RNase-free work environment. Always clean pipettes with RNase decontamination solution and use only nuclease-free pipette tips.
   b. In the final cleanup step, elute cfRNA from beads with 8 µL of nuclease-free water. Immediately proceed with library preparation.

2. Using 5 µL of the rRNA-depleted cfRNA, complete library preparation using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina according to manufacturer's recommendations with the following inputs:
   a. For the RNA fragmentation step, a 7-minute incubation at 94 °C is used.
   b. For adapter ligation, a 1:100 dilution is used.
   c. Use unique NEBNext Multiplex Oligos per sample so that libraries can be pooled for sequencing.
   d. A total of 15 PCR cycles are used for enrichment step.
   e. In the final library cleanup step, the purification beads are eluted with 23 µL of 0.1X TE Buffer.
3. Quantify the finished libraries using the NEBNext Library Quant Kit for Illumina according to manufacturer's recommendations.
   a. **Note 1: Library prep yield is directly proportional to the starting amount of cfRNA. Unlike non-stabilizing legacy tubes, such as EDTA tubes, the Streck RNA Complete BCT maintains draw time concentrations of cfRNA thereby limiting changes to the sequencing library as would occur from contaminating cellular RNA during sample storage.**
4. Assess library quality and size using the Agilent High Sensitivity DNA Chip.
   a. The electropherogram should demonstrate a peak around 300 bp to 400 bp. Peaks at 80 bp and 130 bp should be minimal and correspond to primer-dimers and adaptor-dimers, respectively. See **Figure 2** for representative Agilent TapeStation traces of completed libraries.
5. Store completed libraries at -20 °C until use in sequencing reactions.

Figure 2: Qualitative analysis of sequencing libraries



EDTA Tubes | **Streck RNA Complete BCT®**

Purified cell-free RNA samples from both EDTA (legacy) and Streck RNA Complete BCT (2 hours post-draw) were used to generate sequencing library samples amenable to Illumina-based sequencing methods. Adapter ligated and amplified samples were assayed on the Agilent TapeStation using the High Sensitivity DNA kit. Lower and upper markers are present at 25 and 1500 base pairs, respectively. Red arrows denote library peaks which should be prominent within the 300-400 base-pair region. Primer-dimers (not present) and adaptor-dimers (blue arrows) should remain minimal.

Sequencing, Output, Analysis

1. Prepared libraries may be used with any of the Illumina NextSeq, HiSeq, or NovaSeq instruments. Internal validation of libraries prepared at Streck utilize the Illumina NextSeq 550 instrument.
2. Select appropriate Illumina sequencing kit based on sample number and experimental purpose. For Streck in-house mRNA-seq or gene expression profiling with >12 samples, the NextSeq High Output is used according to manufacturer's recommendations.
3. For Streck internal testing and validation studies, all data is stored, aligned, and analyzed on the Illumina cloud-based BaseSpace Sequencing Hub.

a. Alignment options utilized:

   i. TopHat Alignment

   ii. RNA-seq Alignment

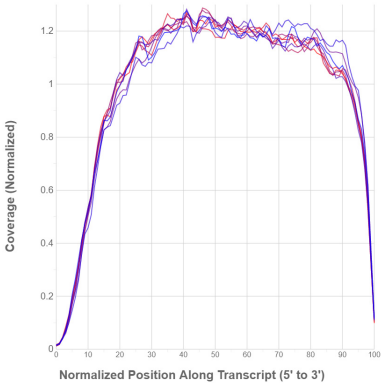
b. Differential expression analysis: DESeq2

c. **Note 2: General multiplex Illumina sequencing requires that all libraries be diluted to the same target concentration in the sequencing pool. For non-stabilized blood samples, increases in total cfRNA lead to increases in library yield, which in turn lead to underrepresentation of specific transcripts (i.e., disease-specific) and overrepresentation of non-specific blood cell breakdown transcripts. The Streck RNA Complete BCT maintains basal (i.e., draw time) gene expression patterns out to 7 days when stored at room temperature.**

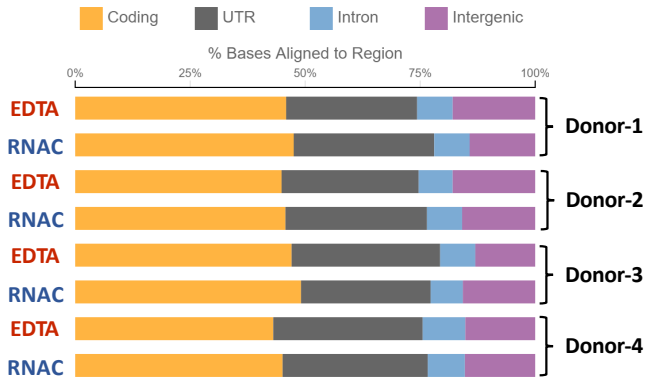
d. Note 3: Libraries generated using this workflow provide uniform coverage across the entire transcript analyzed (**Figure 3**). Additionally, sequenced bases mapping to gene transcripts (i.e., coding regions) are robust and are similar to pre-existing data sets utilizing rRNA depletion protocols. 9, 10, 11

e. Note 4: Draw time expression profiles do not differ between samples drawn into Streck RNA Complete BCT and EDTA blood collection tubes (**Figure 4**). **This demonstrates that the stabilization reagents contained in Streck RNA Complete BCT do not result in immediate changes to the cfRNA pool as would occur with total lysis tubes intended for RNA analysis**.

Figure 3: Streck RNA Complete BCT libraries provide uniform transcript coverage and consistently map to gene coding regions.

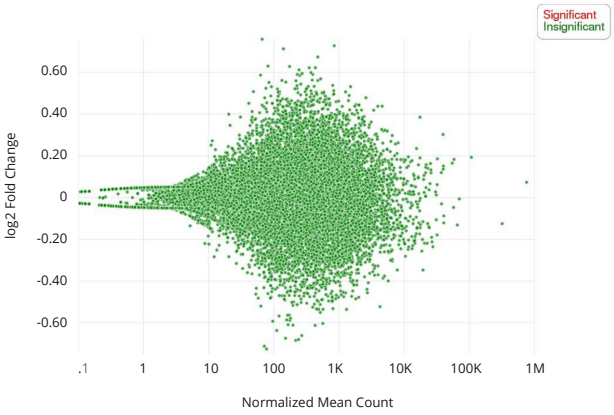


A. Data alignment to the human genome for four healthy donors drawn into EDTA blood collection tubes and Streck RNA Complete BCT. Coverage is uniform along the entire sequenced transcript with no bias observed at 5' or 3' ends.



B. Roughly half of the sequenced bases align to gene coding sequences as expected for a ribosomal RNA depletion-based protocol. Note: Sequence alignment utilized the TopHat alignment application in Illumina BaseSpace.

Figure 4: Draw time gene expression differences are not observed between Streck RNA Complete BCT and EDTA blood collection tubes.



Aligned sequencing data from Figure 3 was queued using the DESeq2 application in Illumina BaseSpace to determine gene transcript differences. No significant changes were observed between tube types suggesting equivalent performance at draw for Streck RNA Complete BCT and the legacy EDTA blood collection tube. Note: compared gene count = 13,561.

References

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