PROTOCOL

truXTRAC® cfDNA Kit – Magnetic Bead

Adaptive Focused Acoustics® (AFA®) -based DNA Extraction & Magnetic Bead-based Purification of Circulating Cell-Free DNA (cfDNA)

Product PN 520221

CONTENTS	PAGE

INTENDED USE

The truXTRAC® cfDNA Kit – Magnetic Bead (PN 520221) is intended for use in research applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

INTRODUCTION

The truXTRAC® cfDNA Kit is designed for the controlled and efficient extraction of cell-free DNA(cfDNA) from plasma prepared from Streck BCT® or EDTA-stabilized blood, and the subsequent magnetic bead-based DNA purification.

truXTRAC cfDNA utilizes Covaris Adaptive Focused Acoustics® (AFA®) to enable Active Extraction of cfDNA from plasma. Active Extraction with AFA mediates dissociation of circulating cell-free DNA from histones, apoptotic bodies and other proteins, including covalently linked DNA-protein complexes, which may be present in Streck BCT stabilized plasma. Active extraction of cfDNA increases the yield and complexity of the extracted cfDNA from plasma, and decreases variations in extraction efficiency due to plasma content.

This protocol is optimized for plasma volumes of 1 ml per AFA treatment. Multiple 1 ml plasma aliquots from the same donor can be processed in parallel and the combined cfDNA eluates can be concentrated (optional).

Important Notes on cfDNA Yield Expectations:

The yield of cfDNA from human plasma can be highly variable and can range from 1 to 20 ng per ml in normal donors, and more than 100 ng per ml in pregnant women, cancer patients, individuals with coronary heart disease, and patients experiencing organ failure and transplant rejection.

Important Notes on Streck BCT stabilized blood and EDTA Blood:

Covaris recommends to collect blood that is being used for cfDNA isolation into EDTA vacutainers or Streck BCT tubes (Streck, PN 218961). Plasma can be isolated from Streck BCT collected blood up to 4 days after venipuncture without any significant contamination from lysed leukocytes [1] provided that manufacturer recommended storage conditions are met. In order to minimize genomic DNA contamination from lysed cells, please follow the Streck Cell-Free BCT recommended centrifugation steps for obtaining plasma.

Plasma from blood collected into EDTA blood collection tubes should be isolated within 4 hours after collection to avoid contamination of plasma with DNA released from lysed blood cells [2].

Note for first time users:
Please contact Covaris at Application Support (ApplicationSupport@covaris.com) if you have any questions.

REVISION HISTORY

Part Number	Revision	Date	Description of change
010374	A	2/18	Release of truXTRAC cfDNA Kit– Magnetic Bead
010374	B	4/18	cfDNA purification step 10 updated
010374	C	6/18	New Rack Definition for ME220

KIT CONTENTS

- Buffer M1 25 ml
- Proteinase K solution 1.25 ml
- Magnetic Bead Suspension 0.5 ml
- Buffer BB2 22 ml
- Buffer WB2 18 ml
- Elution Buffer (BE) 3 ml
- Elution Tubes (DNA LoBind) 24
- milliTUBE 2 ml AFA fiber 24

SDS INFORMATION IS AVAILABLE AT http://covaris.com/resources/safety-data-sheets/

STORAGE

Upon arrival, store the Proteinase K solution and the Magnetic Bead Suspension at 2 to 8 °C.

Store all other kit components at room temperature.

LABORATORY EQUIPMENT, CHEMICALS AND CONSUMABLES TO BE SUPPLIED BY USER

Laboratory Equipment

- Benchtop centrifuge (capable of 1,600 rcf to 16,000 rcf)
- Magnet Stand for 5 ml tubes (e.g. Thermo Fisher, DynaMag™ 5 Magnet, PN 12303D)
- milliTUBE Prep Station (Covaris, PN 500283)

Chemicals

- 100% Isopropanol, ultra-pure (e.g., AmericanBio, PN AB07015)
- 200 proof Ethanol (e.g., AmericanBio, PN AB00515)
- Nuclease-free water (e.g., Ambion, PN AM9930)

Consumables

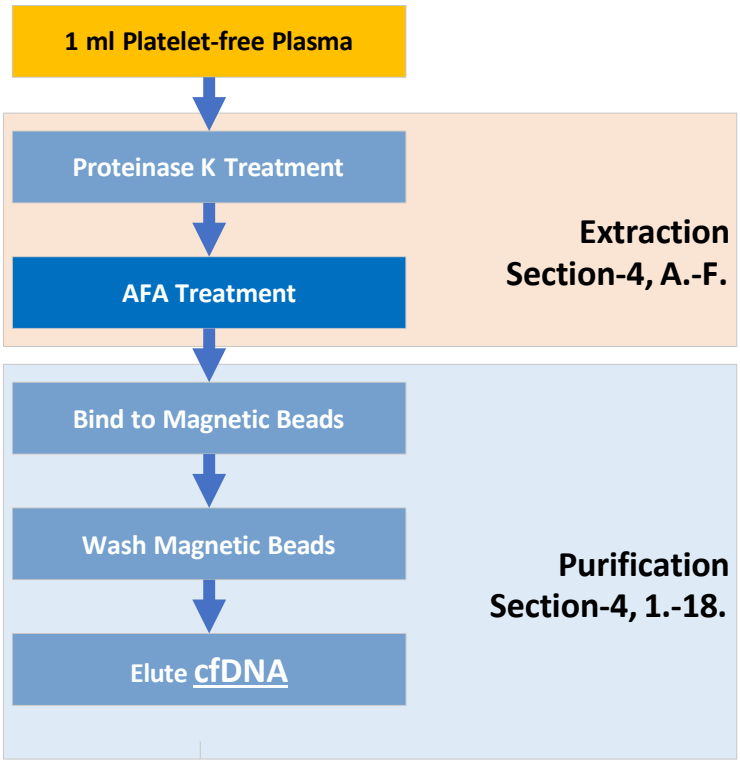
- Eppendorf tubes 5 ml (Eppendorf, PN 0030119401)
- 1.5 ml nuclease free microfuge tubes (e.g., Eppendorf Safe-Lock Tubes, PN 022363212)
- 15 ml conical tubes (e.g., Eppendorf, PN 0030122151)
- 50 ml conical tubes (e.g., Eppendorf, PN 0030122186)

Optional Reagents and Equipment to Concentrate cfDNA in Eluate:

- Bead reagent for PCR cleanup (Ampure XP, Beckman Coulter, PN A63881 or PCRClean DX, Aline Biosciences, PN C-1003)
- Magnet Stand for 1.5-2 ml tubes (e.g. Thermo Fisher, DynaMag-2 Magnet, PN 12321D)

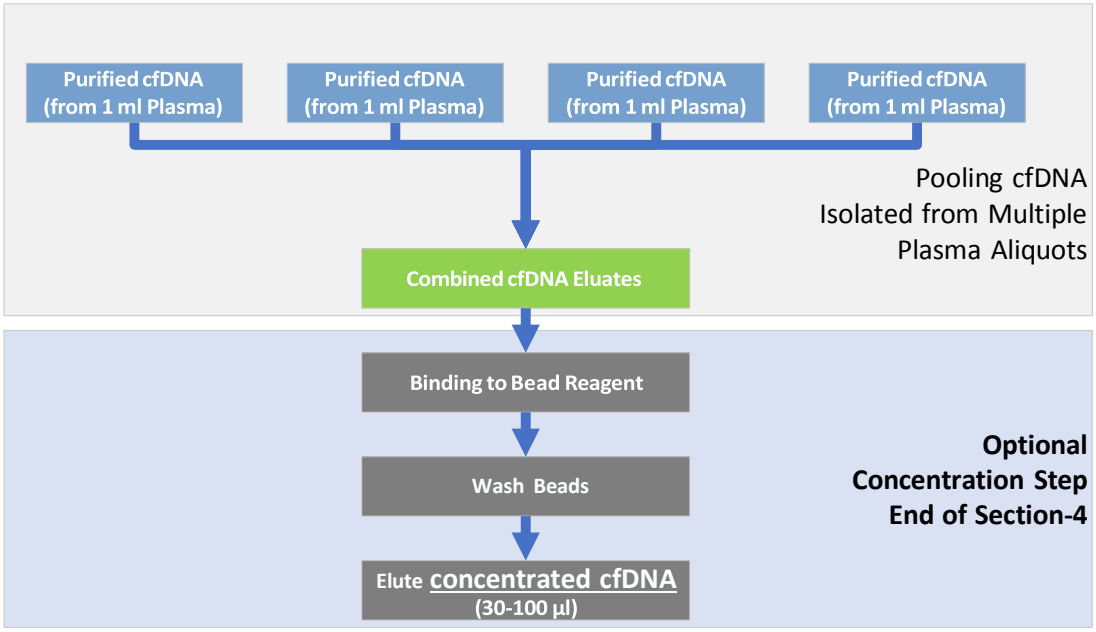
cfDNA Extraction and Purification Workflow

Extraction and purification of cfDNA from 1 ml of Plasma



Concentrate cfDNA isolated from multiple 1 ml Plasma aliquots

(This is an optional procedure)



1 – Preparation of Platelet Poor Plasma

Covaris recommends to collect blood either into EDTA or Streck Cell-Free DNA BCT®.

Both EDTA vacutainers and Streck Cell-Free DNA BCT® collection tubes must be processed according to the published procedure for Streck tubes.

These instructions can be found online:
https://www.streck.com/wp-content/uploads/sync/Collection/Collection_Tubes/Cell-Free_DNA_BCT/01_Instructions_(IFU)/01_Cell-Free_DNA_BCT_IFU.pdf

IMPORTANT: Streck BCT blood should be centrifuged the same day of collection. The plasma can be stored at room temperature for up to five days. For longer term storage, BCT derived plasma can be frozen at -80°C without significant impact on DNA yield.

IMPORTANT: EDTA BCT blood must be centrifuged within 4 hours after collection to minimize contamination of plasma with DNA from lysed blood cells [2].

2 – Preparation of Reagents and Plasma

Please, follow these instructions before starting the cfDNA isolation process.

1. **Plasma**: Before starting the DNA extraction, equilibrate the plasma sample to room temperature by letting it stand on the bench for at least 30 min, or placing it into a waterbath/heatblock at 20°C for 10 min. Processing cold plasma can result in reduced cfDNA yields.

2. **Check Buffer M1 and Buffer WB2**: A precipitate may form during storage. If a precipitate is observed, warm bottles at 50 to 60°C and then gently swirl the liquid until the precipitate is dissolved.

3. **80% Ethanol:** Prepare 80% Ethanol by mixing 4 parts 200 proof Ethanol with 1 part nuclease free water.

4. **Buffer WB2:** Before the 1st use of the kit, add 12 ml of 100% Isopropanol to the Buffer WB2, close bottle tightly and mix by inverting the bottle 5 times. Mark the bottle to indicate that the Isopropanol was added.

5. **Prepare Buffer M1/Proteinase K Mix:** Following the guideline in Table 1, prepare depending on the number of samples (1 ml plasma per sample) being processed and mix by vortexing for 5 seconds. Prepare immediately before use.

Table 1 – Buffer M1/Proteinase K Master Mix

Reagent	Volume for one sample	Volume for N samples
Buffer M1	800 µl	800 µl x 1.1 x N
Proteinase K solution	40 µl	40 µl x 1.1 x N

6. **Prepare Magnetic Bead Suspension/Isopropanol Mix:**
   - Vortex the Magnetic Bead Suspension for at least 15 seconds before preparing the Magnetic Bead Suspension/Isopropanol Mix.
   - Follow Table 2 to prepare the Magnetic Bead Suspension/Isopropanol Mix depending on the number of samples (1 ml plasma per sample) to be processed.Mix by vortexing 5 seconds.

Table 2 – Magnetic Bead Suspension/Isopropanol Mix

Reagent	Volume for one sample	Volume for N samples
Magnetic Bead Suspension	14 µl	14 µl x 1.1 x N
100% Isopropanol	1590 µl	1590 µl x 1.1 x N

3 – FOCUSED ULTRASONICATOR SETUP

For detailed instructions on how to prepare and use your instrument, please refer to the appropriate Covaris Focused Ultrasonicator User Manual.

If you do not detect a Plate Definition on your system, please contact Covaris Technical Support (techsupport@covaris.com)

L-Series

Required Accessory: Rack 24 Place milliTUBE 2 ml (PN500376)

System Setup	
Water Level Set Point	10
Chiller Set Point	18°C
Plate Definition	<500376 24 milliTUBE 2ml>
Rack	Rack 24 Place milliTUBE 2 ml (PN500376)

truXTRAC cfDNA 2 ml Settings	
Peak Incident Power	350 Watts
Duty Factor	30%
Cycles per Burst	600
Temperature (Instrument)	20°C
Treatment Time	120 seconds

After setting up the system, wait until water bath has reached set temperature and the degassing is complete. For water level, use the RUN side of the FILL/RUN water level label when the transducer is submerged.

E220 and E210

Required Accessories: Rack 24 Place milliTUBE 2 ml (PN500376)

System Setup	
Water Level Set Point	10
Chiller Set Point	18°C
Plate Definition	<500376 24 milliTUBE 2ml >
Intensifier	Remove
Rack	Rack 24 Place milliTUBE 2 ml (PN500376)

truXTRAC cfDNA 2 ml Settings	
Peak Incident Power	100 Watts
Duty Factor	30%
Cycles per Burst	600
Temperature (Instrument)	20°C
Treatment Time	120 seconds

After setting up the system, wait until water bath has reached set temperature and the degassing is complete. For water level, use the RUN side of the FILL/RUN water level label when the transducer is submerged.

S-Series

Required Accessory: Holder milliTUBE 2ml (PN500375)

System Setup	
Water Level Set Point	15
Chiller Set Point	18°C
Plate Definition	N/A
Holder	Holder milliTUBE 2ml (PN500375)

truXTRAC cfDNA 2 ml Settings	
Peak Incident Power	100 Watts
Duty Factor	30%
Cycles per Burst	600
Temperature (Instrument)	20°C
Treatment Time	120 seconds

After setting up the system, wait until water bath has reached set temperature and the degassing is complete. For water level, use the RUN side of the FILL/RUN water level label when the transducer is submerged.

ME220

Required Accessories: Rack 4 Place milliTUBE (PN500520), Wave Guide (PN500534)

System Setup	
Rack Definition	<4 milliTUBE-2 AFA Fiber Screw-Cap PN 520186>
Rack	Rack 4 Place milliTUBE (PN500520)
Wave Guide	Waveguide 4 Place (PN500534)

truXTRAC cfDNA 2 ml Settings	
Peak Incident Power	75 Watts
Duty Factor	25%
Cycles per Burst	600
Temperature (Instrument)	20°C
Treatment Time	120 seconds

Position the waveguide into place in the water bath. Wait until water bath has reached set temperature. Load samples into rack and place into the rack holder.
If the system was turned off it is recommended to wait 30 minutes after the temperature set point was reached before sample processing.

M220

Required Accessories: Holder XTU (PN500414), Insert milliTUBE 2 ml (PN500591)

System Setup	
Plate Definition	N/A
Holder	Holder XTU (PN500414)
Insert	Insert milliTUBE 2 ml (PN500591)

truXTRAC cfDNA 2 ml Settings	
Peak Incident Power	50 Watts
Duty Factor	30%
Cycles per Burst	600
Temperature (Instrument)	20°C
Treatment Time	120 seconds

Position Holder XTU and Insert into place and fill the water bath until the water level reaches the top of the holder. After setting up the system, wait until water bath has reached set temperature.

4 - cfDNA EXTRACTION FROM PLASMA AND MAGNETIC BEAD PURIFICATION

AFA-ENHANCED cfDNA EXTRACTION (STEPS A. TO E.)

A. Ensure that the Covaris Focused-ultrasonicator is set up correctly.
Refer to the respective Table in Section-3.

B. Open the milliTUBE and add 1 ml of platelet poor plasma equilibrated to room temperature.

C. Add 840 µl of freshly prepared Buffer M1/Proteinase K Master Mix into each milliTUBE and cap the tube. Mix by inverting 10 times.

D. Incubate the plasma sample in the milliTUBE at 20-25°C for 15 minutes.

IMPORTANT: If incubating on the benchtop, make sure that room temperature is not below 20°C.

E. Place the milliTUBE in the appropriate Covaris Holder/Rack, and process the samples using the truXTRAC cfDNA 2ml Settings specified in the respective table (Section-3).

Note: If automated cfDNA Purification on the KingFisher™ Duo Prime is desired download the Addendum "KingFisher Duo Prime Purification in combination with the cfDNA kit" and the BindIt Files and skip all following steps:

Addendum: **http://covaris.com/wp-content/uploads/pn_010443.pdf**

BDZ Files: **http://covaris.com/wp-content/uploads/M020084.zip**

cfDNA PURIFICATION (STEPS 1. TO 15.)

1. Retrieve the milliTUBE from the Covaris inctrument, open the tube and transfer the AFA-treated plasma into a 5 ml Eppendorf tube.
2. Add 710 µl Buffer BB2 to the AFA-treated plasma, cap the 5 ml Eppendorf tube and vortex for 10 seconds.
3. Vortex the Magnetic Bead Suspension/Isopropanol Mix for 10 seconds (do not centrifuge after vortexing) to ensure that the beads are distributed evenly, and transfer 1.6 ml of the Mix to the sample. Cap the tube and mix by vortexing for 10 seconds.

IMPORTANT: Steps 1. and 2. must be done sequentially, with thorough mixing after each addition. DO NOT MIX ALL THE COMPONENTS AT THE SAME TIME.

4. Place the 5 ml Eppendorf tube on the magnet stand and let sit for 5 minutes.
5. With the tube still situated on the magnet stand, carefully remove and discard the supernatant without disturbing the beads.
6. Remove tube from the magnet stand and add 1 ml Buffer WB2 to the 5 ml Eppendorf tube.
7. Cap the tube and vortex for 10 seconds until all of the beads are resuspended.
8. Place the 5 ml Eppendorf tube on the magnet stand and let it sit for 5 minutes.

9. With the tube still on the magnet stand, carefully remove and discard the supernatant without disturbing the beads.
10. Leave the tube on the magnet stand and pipet 1 ml of 80% Ethanol into the tube without disturbing the bead pellet. Ensure that all beads submerged in the liquid.
11. Incubate for 30 seconds.
12. With the tube still on the magnet stand, carefully remove and discard the 80% Ethanol supernatant without disturbing the pellet.
13. Repeat steps 9 to 11.
14. With the tube still on the magnet stand, remove as much of the Ethanol as possible. Use a 20 to 200 µl pipettor to remove the remaining liquid from the bottom of the tube.
15. Open the tube and dry the beads at room temperature for 15 minutes while the open tube is on the magnet stand.

IMPORTANT: Make sure that all of the Ethanol has evaporated before continuing with elution. Residual Ethanol can inhibit the elution and impact downstream applications such as PCR.

Elution of cfDNA (Steps 16. to 19.)

16. Remove the tube from the magnet stand.
17. Pipet **35 to 50 µl** Elution Buffer BE* into the tube and wash the bead pellet from the tube wall into the bottom of the tube. Mix the beads thoroughly by pipetting up and down until the beads are evenly re-suspended, at least 20 times.
18. Place the tube on the magnet stand and incubate for one minute.
19. Using a 20-200 µl pipette and tip, transfer the eluate to a clean Elution Tube (DNA LoBind) without transferring beads.

    The eluate can also be transferred into Eppendorf DNA LoBind plates (not included).

* Composition of Elution Buffer BE: 5 mM TrisCl pH 8.5

OPTIONAL CONCENTRATION OF THE CFDNA ELUATE FROM MULTIPLE 1 ML EXTRACTIONS (MAGNETIC BEAD BASED PROTOCOL)

If cfDNA extraction from a larger sample volume is desired, the sample can be processed in several 1 ml aliquots using the method above. The cfDNA from each 1 ml plasma aliquot will be eluted in 35 to 50 µl Buffer BE.

To achieve a higher concentration of cfDNA, the eluates then can be pooled and concentrated using a bead reagent for PCR cleanup. The following is the protocol for *PCRClean DX* (Aline Biosciences, PN C-1003).

a. Mix *PCRClean DX* by vortexing thoroughly before adding to samples. Do not spin down after vortexing.

b. In a 2 ml microcentrifuge tube, add *PCRClean DX* reagent at 1.8 fold the volume of the combined eluate.

   Example:
   If the combined eluate volume from 4 x 1.0 ml plasma extraction and purifications is **200** µl, Add 1.8 x **200** µl = 360 µl *PCRClean DX* reagent.

   IMPORTANT: If the cfDNA contains DNA fragments smaller than 100 base pairs, a volume ratio of *PCRClean DX* reagent to sample of 2 or higher may be required to recover all of the DNA.

c. Mix thoroughly by pipetting up and down 10 times and incubate at room temperature for 5 minutes.

d. Transfer the microcentrifuge tube to athe magnet stand and wait 5 minutes.

e. With the tube on the magnet, carefully remove the supernatant using a 200 µl pipette without disturbing the magnetic bead pellet. Discard the supernatant. Repeat until supernatant is completely removed.

f. With the tube on the magnet stand, add 1 ml 80% Ethanol without disturbing the pellet of magnetic particles and wait 30 seconds.

g. With the tube on the magnet stand, carefully remove and discard the 80% Ethanol without disturbing the magnetic bead pellet.

h. Repeat steps f. and g.

i. Remove as much of the 80% Ethanol as possible.

j. Open the tube and allow magnetic beads to dry for 5 minutes open on the magnet stand.

k. Remove the tube from the magnet.

l. Add **30 to 100 µl** Elution Buffer BE (provided in the cfDNA kit) to the tube.

m. Using the pipette, wash the bead pellet from the tube wall into the bottom of the tube. Mix the beads thoroughly by pipetting up and down until the beads are evenly re-suspended, at least 10 times.

n. Place the tube on the magnet and wait for one minute.

o. Transfer the eluate to a clean microcentrifuge tube (DNA LoBind) without disturbing the bead pellet and store the concentrated cfDNA appropriately.

5 - TIPS FOR DETERMINING QUALITY AND QUANTITY OF THE PURIFIED cfDNA

1. Determining the yield and purity of isolated DNA

To determine cfDNA yield, Covaris recommends to use qPCR as fluorometric-based assay dyes (e.g., Qubit) do not bind efficiently to the short cfDNA fragments, and absorbance-based assays (e.g., Nanodrop) lack the sensitivity to accurately assess DNA concentrations at such low concentrations.

Published qPCR primers [3] to amplify two sized amplicons, Alu115 (115 bp) and Alu247 (247 bp) are recommended (Table 3).

The use of a hotstart polymerase is recommended. With the Fast SYBR Green Mastermix (ThermoFisher, PN 4385614), the following conditions for a 2 step PCR reaction can be used: After the initial denaturation, 5 seconds denaturation at 95°C and 30 seconds annealing and extension at 61°C with 2 µl sample volume in a 20 µl PCR reaction.

Table 3 – ALU Primers

Amplicon	Primer Sequence
Alu115	forward 5'-CCTGAGGTCAGGAGTTCGAG-3' reverse 5'-CCCGAGTAGCTGGGATTACA-3'
Alu247	forward 5'-GTGGCTCACGCCTGTAATC-3' reverse 5'-CAGGCTGGAGTGCAGTGG-3'

2. Determining the Integrity of cfDNA by Capillary Electrophoresis

Figure A shows an example of cfDNA that was isolated from plasma prepared from whole blood collected into Streck BCT. The electropherogram shows the expected mono-nucleosomal fraction (peak at 165bp, as well as di- and tri-nucleosomal fractions (340 – 520bp).

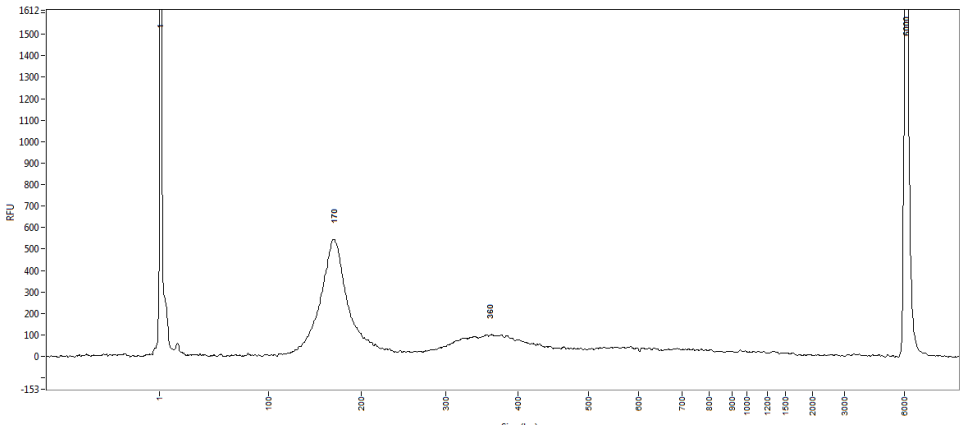


Figure A: A 4 µl aliquot of the total cfDNA eluate (50 µl) was analyzed on a Fragment Analyzer (Advanced Analytical Technologies Inc., Ankeny, IA) using the High Sensitivity NGS Fragment Analysis Kit (PN DNF-474-0500). Injection time was 90 seconds.

REFERENCES

1. Inga Medina Diaz, Annette Nocon, Daniel H. Mehnert, Johannes Fredebohm, Frank Diehl, Frank Holtrup: Performance of Streck cfDNA Blood Collection Tubes for Liquid Biopsy Testing, PLoS One. 2016 Nov 10;11(11):e0166354. doi: 10.1371/journal.pone.0166354. eCollection 2016.

2. S.E. Norton, J.M. Lechner, T. Williams, M.R. Fernando: A stabilizing reagent prevents cell-free DNA contamination by cellular DNA in plasma during blood sample storage and shipping as determined by digital PCR, Clinical Biochemistry, Volume 46, Issue 15, October 2013, Pages 1561-1565, ISSN 0009-9120

3. Alison S. Devonshire, Alexandra S. Whale, Alice Gutteridge, Gerwyn Jones, Simon Cowen, Carole A. Fo, Jim F. Huggett: Towards standardisation of cell-free DNA measurement in plasma: controls for extraction efficiency, fragment size bias and quantification, Anal Bioanal Chem (2014) 406:6499–6512.