PROTOCOL

truXTRAC™ cfDNA for Plasma - Column Purification Kit (24)

Adaptive Focused Acoustics™ (AFA) -based DNA Extraction & Column-based Purification of Circulating Cell-Free DNA (cfDNA)

Product PN 520234

Contents

INTENDED USE

The truXTRAC™ cfDNA for Plasma - Column Purification Kit 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 cfDNA from plasma prepared from Streck BCT® or EDTA-stabilized blood, and the subsequent spin-column based DNA purification.

The Covaris Adaptive Focused Acoustics™ (AFA™)-enabled truXTRAC cfDNA extraction system 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 0.9 ml.

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 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 immediately 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@covarisinc.com) if you have any questions.

REVISION HISTORY

Part Number	Revision	Date	Description of change
010382	A	3/17	Release of truXTRAC cfDNA – Column Purification kit
010382	B	5/17	Fix the wrong product number and typo issues

KIT CONTENTS

Conditioning Buffer (CB)	1 ml
Proteinase K (PK Solution)	1.25 ml
Binding Buffer (BB2)	11 ml
BW Buffer	15 ml
B5 Buffer	5 ml
Elution Buffer (BE)	3 ml
Purification Columns	24
Collection Tubes	50
Elution Tubes (DNA LoBind)	24
milliTUBE 1ml AFA fiber	24

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

STORAGE

Store the Proteinase K solution at 2-8 °C.

Store all other kit components at room temperature.

SUPPLIED BY USERS

Covaris Instruments and Parts

Required parts						
Focused-ultrasonicator	**LE220**	**E220 & E210**	**E220 evolution**	**S-Series**	**ME220**	**M220**
Rack/ Holder	Rack 24 Place milliTUBE 1ml PN500368	Rack 24 Place milliTUBE 1ml PN500368	Rack E220e 4 Place milliTUBE 1ml PN500431	Holder milliTUBE 1ml PN500371	Rack 4 Place milliTUBE 1ml PN500520 & Waveguide PN500534	Holder XTU PN500414 & Insert XTU 500422 **(*)**
Intensifier	NA	Yes	Yes	NA	NA	NA
Optional parts						
Accessories	milliTUBE Prep Station 4 Place PN 500338					

(*) Holder PN500348, although discontinued, can also be used. This holder does not require an insert

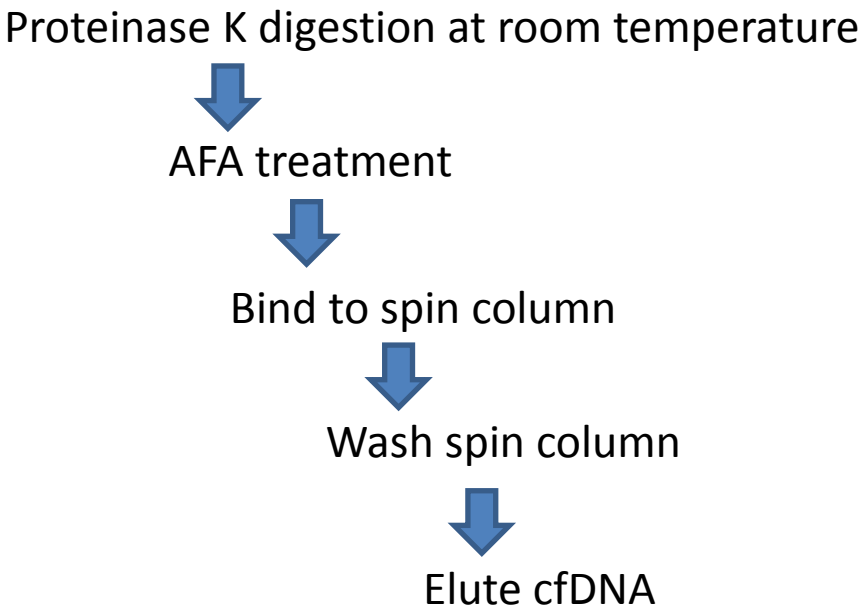
Other supplies (not provided in this kit):

- Plasma preferably obtained from whole blood collected into Cell-Free DNA BCT Collection Tubes (Streck, PN 218961)
    - For plasma preparation: Benchtop centrifuges capable of 1,600 rcf and 16,000 rcf per manufacturer's protocol
- 100% Isopropanol, ultra-pure (e.g., AmericanBio, PN AB07015)
- Ethanol, absolute alcohol, 200 proof (e.g., AmericanBio, PN AB00515)
- Nuclease-free water (e.g., Ambion, PN AM9930)
- 2.0 mL Nonstick, Nuclease free Microfuge Tubes (e.g., Thermo Fisher Scientific, PN AM12475)
- 1.5 ml Nuclease free Microfuge Tubes (e.g., Eppendorf Safe-Lock Tubes, PN 022363212)
- Microcentrifuge with 16,000 x g capability.
- Dry block heater (e.g., Eppendorf ThermoMixer C, PN 2231000269) for 1.5 mL tubes, capable of heating to 56°C.
- 5 ml centrifuge tubes (e.g., Eppendorf Tubes 5 ml, PN 0030119401)* or
- 15 ml conical tubes (e.g., Eppendorf Conical Tubes 15 ml, PN 0030122151)*

**If processing of the entire plasma input volume is desired.*

PROCEDURE WORKFLOW OVERVIEW

EXTRACT FROM 0.9 ML OF PLASMA



1 - PREPARATION OF PLASMA FROM STRECK BCT STABILIZED BLOOD

For cfDNA extraction, Covaris recommends to collect blood into Streck Cell-Free DNA BCT®. Refer to the manufacturer's instructions for the most up-to-date processing procedure.

NOTE: For best results, Streck BCT blood is 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.

Instructions for use of Streck tubes for preparation of plasma:

https://www.streck.com/resources/Cell_Stabilization/Cell-Free_DNA_BCT/01_Instructions_(IFU)/01_IFU_Cell-Free_DNA_BCT_IFU.pdf

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

2 – PREPARATION OF REAGENTS

1. **Check Conditioning Buffer and Wash Buffer**: 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.
2. **Add Ethanol to B5 Buffer:** Add 20 ml of ethanol (>96%) to B5 Buffer concentrate, mix thoroughly by inverting the bottle several times and mark the label on the cap. After preparation, B5 Buffer can then be stored for one year at room temperature. The cap should be closed tightly when not in use to prevent evaporation of ethanol.
3. **Prepare Conditioning Buffer Master Mix:** Prepare according to the table below depending on the number of samples (0.9 ml plasma per sample) being processed.

Table 1 – Conditioning Buffer master mix

Total Number of samples	Conditioning Buffer volume *	PK Solution Volume *
1	**26** µl	**44** µl
6	**156** µl	**264** µl
12	**312** µl	**528** µl
24	**624** µl	**1056** µl
x	**X 26** µl	**X 44** µl

* calculations include 10% excess

3 – FOCUSED-ULTRASONICATOR SETUP

1. **S, E, or LE-Series Focused-ultrasonicators:**
   Set up the instrument as shown in table below. Wait for the water to reach temperature and to degas.

Table 2 - Focused-ultrasonicator setup

Instrument	Water level*	Chiller Set Point	Intensifier PN500141	Plate definition**	Holder or Rack	Wave Guide
S-Series	**8**	**18°C**	**NA**	NA	PN500371	NA
E220	**0**	**18°C**	**Yes**	Rack 24 Place milliTUBE 1ml PN500368	PN500368	NA
E220 evolution	**0**	**18°C**	**Yes**	Rack E220e 4 Place milliTUBE 1ml PN500431	PN500431	NA
LE-Series	**0**	**18°C**	**NA**	Rack 24 Place milliTUBE 1ml PN500368	PN500368	NA
M220	**NA**	**20°C**	**NA**	NA	PN500414 & 500422 insert	NA
ME220	**NA**	**20°C**	**NA**	ME220 Rack 4 Place milliTUBE 1 ml	PN500520	PN500534

***** Use RUN side of Fill/Run scale
**If you do not see a plate definition on your system, please contact Covaris technical support at TechSupport@covarisinc.com

2. **M220 Focused-ultrasonicators**:
   Put the Holder PN500414 and the Insert PN500422 (or the discontinued Holder PN500348 without insert) in place and fill the water bath until the water reaches the top of the holder. Allow system temperature to reach 20°C.
3. **ME220 Focused-ultrasonicators**:
   Put the Wave Guide PN500534 into place in the water bath. Load samples into rack PN500520 and place into the rack holder.

For detailed instructions on how to prepare your instrument, please refer to the respective User Manual.

4 – DNA extraction

NOTE: Set heating source to 56°C and preheat the required volume of Buffer BE in a 1.5 mL microfuge tube: (number of samples x 50 µl x 1.1)

1. Open the milliTUBE and add 900 µl of platelet poor plasma.
2. Add 64 µl of freshly prepared Conditioning Buffer Master Mix into each milliTUBE, and mix by pipetting up and down 5 times, or by inverting the tube 10 times after capping the tube.
3. Incubate at room temperature (20-23°C) for 15 minutes.
4. Place the milliTUBEs in the appropriate holder/rack, and process the samples using the settings below in Table 3.

Table 3 – Covaris AFA Processing Conditions

System	Duty Factor	Peak Incident Power	Cycles per burst	Treatment Time	Temperature (Instrument)
S220 or E220	**10%**	**100 Watts**	**200**	**60 sec**	**20°C**
M220	**15%**	**50 Watts**	**200**	**60 sec**	**20°C**
LE220	**20%**	**350 Watts [(1)]**	**200**	**60 sec**	**20°C**
ME220	**15%**	**75 Watts**	**200**	**60 sec**	**20°C**

[(1)] As Covaris LE220 process multiple samples at a time, its PIP is distributed across microTUBEs, and power received by individual microTUBE stays within the 200 W limit.

Depending of the tube format chosen for the purification, two options are available:

Option 1 - 2ml centrifuge tube format from 0.8 ml conditioned plasma sample

Option 2 - 5 ml or 15 ml tube format from entire conditioned plasma sample

Option 1: Extraction of cfDNA from 0.8 ml plasma in 2 ml microcentrifuge tube format

a. Transfer 800 µl of the conditioned plasma into a clean 2.0 ml microcentrifuge tube.
b. Add 315 µl Binding Buffer (BB2) to the sample and vortex for 5 seconds.
c. Add 700 µl 100% Isopropanol to the sample and vortex for 5 seconds.
d. Assemble a Purification Column on top of a Collection Tube.
e. Aliquot 620 µl of the sample onto the Purification Column.
f. Spin the Column/Tube assembly at 11,000 x g for 30 seconds. Discard the flow-through and assemble the column back onto the Collection Tube.
g. Repeat steps **e** and **f** for a total of 3 times until entire sample has passed through the same Purification Column.
h. Proceed to step 5: 1st wash

Option 2: Extraction of cfDNA from complete plasma sample in 5 ml or 15 ml tube format

a. Transfer 960 µl of the conditioned plasma into a clean 5 ml microcentrifuge tube or 15 ml conical tube.
b. Add 378 µl Binding Buffer (BB2) to the sample and vortex for 5 seconds.
c. Add 840 µl 100% Isopropanol to the sample and vortex for 5 seconds.
d. Assemble a Purification Column on top of a Collection Tube.
e. Aliquot 550 µl of the sample onto the Purification Column.
f. Spin the Column/Tube assembly at 11,000 x g for 30 seconds. Discard the flow-through and assemble the column back onto the Collection Tube.
g. Repeat steps **e** and **f** for a total of 4 times until entire sample has passed through the same Purification Column.
h. Proceed to step 5: 1st wash

5. **1st wash:** Add 500 µl BW Buffer onto the Column. Spin the Column/Tube assembly at 11,000 x g for 60 seconds.
6. Discard the flow-through and place the Column back in the Collection Tube.
7. **2nd wash:** Add 600 µl prepared B5 Buffer onto the Column. Spin the Column/Tube assembly at 11,000 x g for 60 seconds.
8. Discard the flow-through and place the Column back in the Collection Tube.

9. **3rd wash:** Add 600 µl 100% ethanol onto the column and spin the Column/Tube assembly at 11,000 x g for 60 seconds.
10. **Dry Column:** Transfer Column to a fresh Collection Tube and centrifuge at 16,000 x g for 3 minutes.
11. Transfer Purification Column into a provided 1.5 ml elution tube.
12. Place Column/Tube assembly with opened Column in a 56°C heat block for 10 minutes.
13. **Elute cfDNA:** Remove the Column/Tube assembly from the heat block and add 50 µl of pre-warmed Buffer BE (56°C) to the center of the column. Incubate at room temperature for 3 minutes.
14. Spin the Column/Tube assembly at 16,000 x g for 60 seconds to collect the cfDNA sample. Discard the column.

ADDITIONAL NOTES

1. Determining the yield and purity of isolated DNA:

To determine cfDNA yield, we recommend to use qPCR since 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 amounts.

We recommend qPCR primers [3] to amplify two sized amplicons, Alu115 (115 bp) and Alu247 (247 bp). See Table 4 below for primer sequence information.

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.

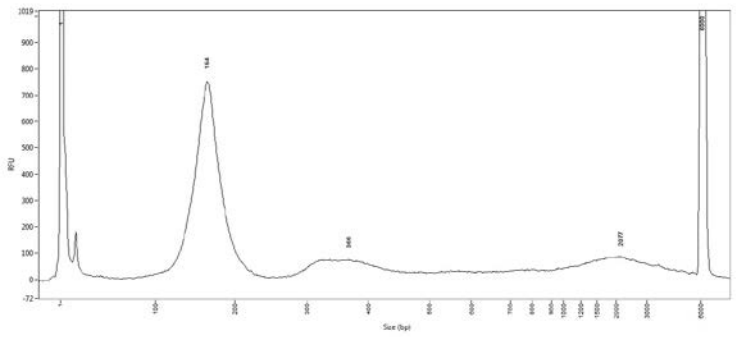
Table 4 – ALU Primers

Amplicon	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:

Shown is an example of cfDNA that was isolated from plasma prepared from whole blood collected into Streck BCT. A 2 µl aliquot of the total Spin Column 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.

The electropherogram shows the expected nucleosomal fraction, peaking at 165 bp, as well as a higher molecular weight fractions.



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