truXTRAC® FFPE total Nucleic Acid Plus Kit - Column

Adaptive Focused Acoustic® (AFA) -based
sequential RNA and DNA extraction from FFPE tissues
using column-based purification

PN 520252

INTENDED USE

The truXTRAC® FFPE total NA (Nucleic Acid) Plus Kit is intended for research use only. This product is not intended for the diagnosis, prevention, or treatment of any disease.

INTRODUCTION

The truXTRAC® FFPE total NA Plus Kit is designed for efficient and parallel extraction of total nucleic acids (RNA and DNA) from Formalin Fixed, Paraffin Embedded (FFPE) tissue samples using Covaris Adaptive Focused Acoustics (AFA®).

AFA-energetics® enables the active removal of paraffin from FFPE tissue samples in an aqueous buffer, allowing simultaneous tissue rehydration. Compared to traditional passive, chemical-based methods of paraffin removal, this mechanical process is not as limited by the thickness of FFPE tissue sections. Thus, the use of AFA enables input of thicker FFPE sections, which results in increased yields of nucleic acids, whilst minimizing the degradation of nucleic acids exposed at the FFPE section surface. The truXTRAC® process results in high yields of high-quality RNA and DNA well suited for analytical methods such as next-generation sequencing (NGS) or qPCR/RT-qPCR.

This protocol is optimized for FFPE sections up to a total of 40-45 µm in thickness. For samples of smaller input sizes, the truXTRAC total NA Kit (PN 520220) may be used for extraction and purification of DNA and RNA from FFPE samples

Important Notes on FFPE Samples:

The yield of DNA and RNA from FFPE tissue blocks can be highly variable. Factors such as fixation time, size and thickness of the sections, the ratio of tissue to paraffin, the type of tissue, and the age and storage conditions of the FFPE block are the main causes for this variability.

The quality of DNA and RNA isolated from FFPE samples can also be highly variable. During the fixation process, DNA and RNA are cross-linked to proteins and other nucleic acid molecules to varying degrees. The nucleic acid fragment or strand length isolated from FFPE samples is generally shorter as compared to nucleic acids that are isolated from fresh or frozen tissues [1]. This is particularly evident in older FFPE sample blocks or sample blocks stored at elevated temperatures.

Note for first time users:

Given the highly variable yield of total NA from FFPE tissue blocks, we recommend using FFPE blocks that have been well characterized for yield and quality for initial testing of the truXTRAC® FFPE total NA Plus kit. Ideally, nucleic acids should be extracted immediately after sectioning of the samples.

If you require any help with this product please check the FAQs found on our website or contact Covaris Application Support at ApplicationSupport@covaris.com.

REVISION HISTORY

Part Number	Revision	Date	Description of change
010435	A	2/13/18	Initial release of truXTRAC FFPE Total NA Plus Kit - Column

KIT CONTENTS

○ NA Plus Tissue Lysis Buffer	25 ml
○ Proteinase K (PK) Solution	3.5 ml
○ Total NA Plus B1 Buffer	40 ml
○ RNA Wash Buffer	10 ml
○ RNA Elution Buffer	3 ml
○ BW Buffer	15 ml
○ Total NA Plus B5 Buffer	7 ml
○ BE Buffer	7.5 ml
○ RNA Purification Columns	25
○ DNA Purification Columns	25
○ Collection Tubes	50
○ RNA Elution Tubes	25
○ microTUBE-500 AFA Fiber Screw-Cap FFPE	25

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

STORAGE

Upon kit arrival, store the Proteinase K solution at 2-8 °C.

Store all other kit components at room temperature.

LABORATORY EQUIPMENT, CHEMICALS AND CONSUMABLES TO BE SUPPLIED BY USER

Required Laboratory Equipment and Accessories

- Microcentrifuge (16,000 x g capability)
- microTUBE and microcentrifuge tube incubator
- microTUBE-500 Centrifuge and Heat Block Adapter (Covaris PN500503)

IMPORTANT: Covaris strongly recommends dry block heaters to be used for all steps requiring incubation at 56°C or 80°C. Covaris microTUBEs should be used in conjunction with Covaris Centrifuge and Heat Block microTUBE Adapters (PN500503).

Examples of dry block heaters are the Eppendorf ThermoStat-C (PN5383000027) in combination with 1.7 or 2mL SmartBlock (PN5362000036 or 5362000035) and ThermoTop (PN5308000003); alternatively the VWR Advanced Mini Dry Block Heater with Heated Lid (PN10153-348) in combination with MiniBlock 2ml Tubes (PN10153-366) is recommended.

Chemical and Enzymes

- 100% isopropanol, ultrapure (e.g. AmericanBio, PN AB07015)
- 200 proof ethanol (e.g., AmericanBio, PN AB00515)
- Nuclease-free water (e.g. Ambion, PN AM9930)
- Optional DNase-free RNase A (10 mg/ml) (e.g. Thermo Scientific, PN EN0531)

Consumables

- 1.5 ml nuclease free microfuge tubes (e.g., Eppendorf Safe-Lock Tubes, PN 022363212)
- 2 ml nuclease free microfuge tubes (e.g., Eppendorf Safe-Lock Tubes, PN 022363352)

Optional Supplies for FFPE Sample Collection & Processing

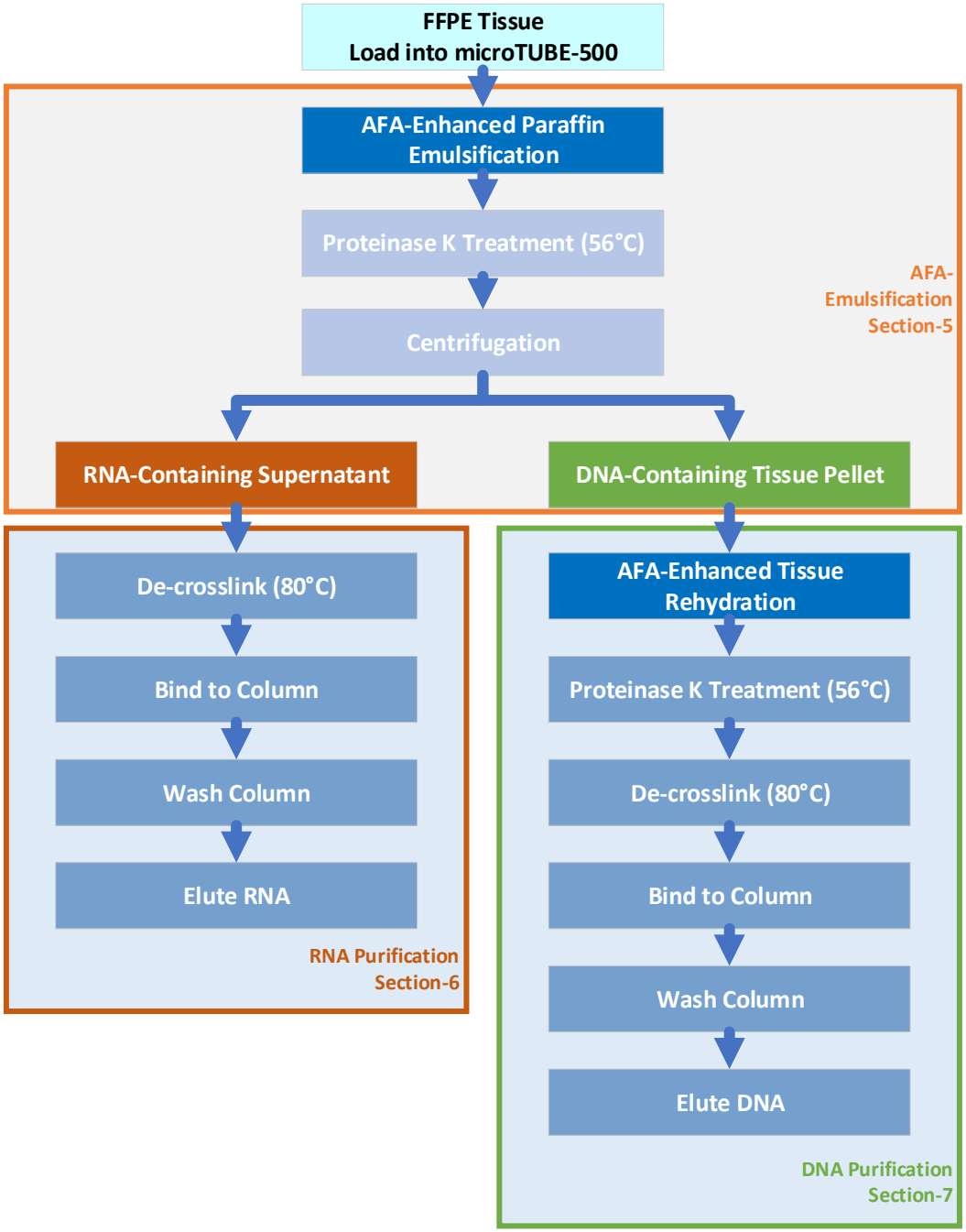
- FFPE tissuePICK (Covaris, PN520163)
- tissuePICK Forceps (5) (Covaris, PN 520164)
- FFPE sectionPICK (Covaris, PN520149)
- FFPE sectionWARMER (Covaris, PN500403)

FFPE tNA Extraction And Purification Workflow

Using AFA, FFPE tissue sections are emulsified in Total NA Plus Tissue Lysis Buffer in the presence of Proteinase K, followed by incubation at 56°C for a short duration. This results in release of RNA and minimizes over-digestion of the tissue and loss of genomic DNA.

The RNA-containing supernatant is separated from the DNA containing tissue by a centrifugation step. RNA is then de-crosslinked and purified over a spin column.

In parallel, DNA is released from the tissue by AFA-enhanced Proteinase K digestion, following a de-crosslinking step. DNA is then column-purified.



1 – FFPE Sample Input Requirements and Guidelines

The truXTRAC process is highly efficient at removing paraffin, even from relatively thick FFPE sections, while simultaneously rehydrating the tissue.

WARNING: Excess paraffin may adversely affect the yield and quality of DNA and RNA extracted from FFPE specimens. If possible, it is strongly recommended to trim off any excess paraffin before sectioning a FFPE tissue block.

The rules and guidelines in the table below are very important as the FFPE section thickness, total mass, and paraffin to tissue ratio will greatly influence the quality and quantity of extractable nucleic acids.

	FFPE Sections from Slides		FFPE Sections from Blocks	
FFPE Sample collected with	Scalpel or tissuePICK	Scalpel or sectionPICK	Microtome (scroll)	Coring Device (core)
FFPE Sample Dimensions & sections per microTUBE-500	Up to 4 4 - 10 µm thick picks	Up to 4 7 - 10 µm thick picks	up to 4 7 - 10 µm tick x 10 mm long or up to 3 15 µm thick x 10 mm long	up to 2 15 gauge diameter x 10 mm long

WARNING: The total mass of FFPE sample per microTUBE-500 must be between 2 to 12 mg. Lower amounts may result in insufficient RNA and DNA yield. Higher amounts may impact purification efficiencies due to spin column clogging.

IMPORTANT: For optimal tissuePICK and sectionPICK performances, the tissue section should be mounted on uncoated slides. tissuePICK and sectionPICK should always be used in conjunction with a Covaris sectionWARMER.

FFPE Tissue Preparation Recommendations

The yield and quality of nucleic acids extracted from FFPE samples is highly dependent on tissue collection and paraffin embedding procedures. For good yields of high quality DNA/RNA do not exceed a fixation time of 24 hours, use 4% neutral buffered formalin solution and fix tissue sample as quickly as possible after collection.

2 – Preparation of Reagents

Please, follow these instructions before starting the FFPE tNA isolation process.

1. **RNA Wash Buffer:** The RNA Wash Buffer is supplied as a concentrate. Before initial use, add 40 ml of 200 proof Ethanol to the RNA Wash Buffer concentrate.
   Close the bottle and mix by inverting 5 times. Mark the bottle label accordingly.
   After preparation, the RNA Wash Buffer can be stored for up to one year at room temperature.
   Minimize the number of times the bottle is opened to avoid evaporation of Ethanol.

2. **Total NA Plus B5 Buffer**: The Total NA Plus B5 Buffer is supplied as a concentrate. Before initial use, add 28 ml of 200 proof Ethanol to the B5 Buffer concentrate.
   Close the bottle and mix by inverting 5 times. Mark the bottle label accordingly.
   After preparation, the B5 Buffer can be stored for up to one year at room temperature. Minimize the number of times the bottle is opened to avoid evaporation of ethanol.

3. **Total NA Plus B1 Buffer and Total NA Plus Tissue Lysis Buffer**: Check these buffers visually, as a white precipitate may form during storage. If white precipitate is visible, incubate the buffer bottles at 50 – 60 °C for 5 to 10 minutes before use to dissolve any precipitate.

3 – Heating Element/Dry Block Guidelines

IMPORTANT: Covaris strongly recommends dry block heaters to be used for all steps requiring incubation at 56 or 80°C.
Covaris microTUBEs should be used in conjunction with Covaris Centrifuge and Heat Block microTUBE Adapters (PN500503).

It is important to use an accurate heating source for incubation of microTUBE-500 and microcentrifuge tubes during Proteinase K and de-crosslinking reactions incubations. Lower or higher than the indicated temperatures can significantly impact quality and quantity of purified nucleic acids.

1. Preheat dry block heaters to 58°C and 82°C.

2. Test the temperature of your water bath and heat blocks:
   a. Place a microcentrifuge tube (1.7 or 2 ml) filled with water into the heat block.
   b. Immerse a thermometer into the tube.
   c. Wait until the temperature has reached the plateau.
   d. Adjust the Set-temperature accordingly until the temperature inside the microcentrifuge tube has reached 56°C or 80°C.

4 – FOCUSED-ULTRASONICATOR SETUP

For detailed instructions on how to prepare and use your instrument, please refer to the respective Covaris User Manual. If you do not detect a Plate Definition on your system, please contact Covaris Technical Support (techsupport@covaris.com)

Using the settings provided in the section below, generate **two** programs in the Sonolab software of your system for **1. Emulsification**, and the **2. Tissue Resuspension**. Please note that the processing setting variables can vary depending on Covaris instrument type, so please use the settings specific to your Covaris instrument model.

L-Series

Required Accessory: Rack 24 microTUBE-500 Screw-Cap (PN500452)

System Setup

Water Level Set Point	8
Chiller Set Point	20°C
Plate Definition	<500452 Rack 24 microTUBE-500 Screw-Cap>
Rack	Rack 24 microTUBE-500 Screw-Cap (PN500452)

truXTRAC FFPE microTUBE-500 Settings

Peak Incident Power	450 Watts
Duty Factor	30%
Cycles per Burst	200
Temperature (Instrument)	20°C
Emulsification Treatment Time	300 seconds
Tissue Resuspension Time*	10 seconds

* This time setting is used at a later point in the protocol.

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 microTUBE-500 Screw-Cap (PN500452), intensifier (PN500141)

System Setup

Water Level Set Point	8
Chiller Set Point	20°C
Plate Definition	<500452 Rack 24 microTUBE-500 Screw-Cap>
Intensifier	PN500141
Rack	Rack 24 microTUBE-500 Screw-Cap (PN500452)

truXTRAC FFPE microTUBE-500 Settings

Peak Incident Power	200 Watts
Duty Factor	30%
Cycles per Burst	200
Temperature (Instrument)	20°C
Emulsification Treatment Time	300 seconds
Tissue Resuspension Time*	10 seconds

* This time setting is used at a later point in the protocol.

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 evolution

Required Accessories: Rack E220e 4 microTUBE-500 Screw Cap (PN500484), Intensifier (PN500141)

System Setup

Water Level Set Point	8
Chiller Set Point	20°C
Plate Definition	<500484 Rack E220e 4 microTUBE-500 Screw Cap>
Intensifier	PN500141
Rack	Rack E220e 4 microTUBE-500 Screw Cap (PN500484)

truXTRAC FFPE microTUBE-500 Settings

Peak Incident Power	200 Watts
Duty Factor	30%
Cycles per Burst	200
Temperature (Instrument)	20°C
Emulsification Treatment Time	300 seconds
Tissue Resuspension Time*	10 seconds

* This time setting is used at a later point in the protocol.

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 microTUBE-500 Screw-Cap (PN500449)

System Setup

Water Level Set Point	8
Chiller Set Point	20°C
Plate Definition	N/A
Holder	Holder microTUBE-500 Screw-Cap (PN500449)

truXTRAC FFPE microTUBE-500 Settings

Peak Incident Power	200 Watts
Duty Factor	30%
Cycles per Burst	200
Temperature (Instrument)	20°C
Emulsification Treatment Time	300 seconds
Tissue Resuspension Time*	10 seconds

* This time setting is used at a later point in the protocol.

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 Position microTUBE-500 (PN500525), Wave Guide (PN500534)

System Setup

Rack Definition	<4 microTUBE-500 Screw-Cap PN 520185>
Rack	Rack 4 Position microTUBE-500 (PN500525)
Wave Guide	ME220 Waveguide 4 Place (PN500534)

truXTRAC FFPE microTUBE-500 Settings

Peak Incident Power	75 Watts
Duty Factor	25%
Cycles per Burst	1000
Temperature (Instrument)	20°C
Emulsification Treatment Time	300 seconds
Tissue Resuspension Time*	10 seconds

* This time setting is used at a later point in the protocol.

Position the wave guide 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 XTU (PN500471)

System Setup

Holder	Holder XTU (PN500414)
Insert	Insert XTU (PN500471)

truXTRAC FFPE microTUBE-500 Settings

Peak Incident Power	75 Watts
Duty Factor	20%
Cycles per Burst	200
Temperature (Instrument)	20°C
Emulsification Treatment Time	300 seconds
Tissue Resuspension Time*	10 seconds

* This time setting is used at a later point in the protocol.

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.

5 – Paraffin Emulsification & Tissue Rehydration

1. Set-up the Focused-ultrasonicator following the respective guidelines in Section-4. Use **truXTRAC FFPE microTUBE-500 Emulsification Time** settings.

2. Set-up the dry-heat blocks as explained in Section-3 and verify the block temperatures to be 56°C. Insert the required number of microTUBE-500 Centrifuge and Heat Block Adapters into the heat block.
   Note: it is recommended to set-up another dry block heater for a target temperature of 80°C at the same time, which will be necessary for the de-crosslink steps.

3. Prepare Total NA Plus Tissue Lysis Buffer/Proteinase K Mix in a microcentrifuge tube following instructions in Table 1 below, and mix by inverting 10 times or vortexing for 3 seconds.

NOTE: The Total NA Plus Tissue Lysis Buffer/Proteinase K Mix should be stored at room temperature. Use within 30 min after preparation.

Table 1 – Total NA Plus Tissue Lysis Buffer /Proteinase K Mix

Reagent	Volume for one sample*	Volume for N samples*
Tissue Lysis Buffer	**440** µl	**440** µl x N
Proteinase K Solution	**44** µl	**44** µl x N

* calculation includes 10% excess in final volume

4. Open the microTUBE-500 Screw-Cap and load the FFPE tissue.

5. Add 440 µl Tissue Lysis Buffer/Proteinase K mix.

6. Close the microTUBE tightly with the Screw-Cap.

7. Process the sample in the Focused-ultrasonicator, using the **truXTRAC FFPE microTUBE-500 Emulsification Time** settings (Section-4).

NOTE: It is expected that the solution will turn milky white. Please see the example in Appendix A.

8. Remove the microTUBE-500 from the Focused-ultrasonicator and proceed to step 9.

9. Load the microTUBE into the microTUBE-500 Centrifuge and Heat Block adapter.

10. Incubate for 30 minutes at 56°C, remove microTUBE-500 together with the microTUBE-500 adapter from the heat block, and let cool at room temperature for 3 min.

IMPORTANT: Do not chill on ice.

11. Place microTUBE-500 in the microTUBE-500 adapter with the bar code on the screw cap sleeve facing outward into a microcentrifuge (fixed angle rotor) and centrifuge at 5,000 x g for 15 minutes.

IMPORTANT: microTUBE-500 Centrifuge and Heat Block Adapters must be used. Do not spin faster than 5000 x g. The centrifuge lid may not close and may need to be left off during centrifugation.

12. Open the microTUBE and carefully transfer 400 µl supernatant into a 2 ml microcentrifuge tube.

IMPORTANT: This is a critical step in the workflow. By following these guidelines, the risk of losing DNA-containing tissue will be minimized:

a. **Locate the DNA-containing tissue pellet. It will be located on the same side as the barcode which faces outward during centrifugation.**
b. **Tilt the tube slightly away from the pellet.**
c. **Using a 200 µl pipette with a 200 µl tip, slowly pierce the upper layer containing emulsified wax and carefully aspirate the supernatant while simultaneously lowering the tip following the liquid level. Place the pipette tip towards the tube wall that faces away from the barcode. DO NOT USE WIDE-MOUTH TIPS.**
d. **A cloud of emulsified wax may descend obscuring the pellet. This is normal.**
e. **Leave 20-30 µl of supernatant behind. This will not affect RNA or DNA yield significantly.**

13. Save the DNA-containing tissue pellet for subsequent DNA purification as described in Section-7. The DNA-containing pellet can be stored on ice or at 2–8°C for up to 1 day. For longer periods, store at –15 to –30°C.

Proceed to RNA Purification (Section-6).

Note: It is optional to start RNA Purification while DNA is being incubated during Proteinase K treatment and de-crosslinking.

6 - RNA PURIFICATION

1. Set-up the dry-heat blocks as explained in Section-3 and verify the block temperatures to be 80°C.

2. Incubate the 2 ml microcentrifuge tube with the RNA-containing supernatant at 80°C for 20 minutes. Remove tubes and let cool for 3 minutes.

IMPORTANT: Do not chill on ice.

3. Prepare RNA Purification Columns by inserting them into the Collection Tubes.

4. Add 375 µl Total NA Plus B1 Buffer to the de-crosslinked RNA-containing supernatant and mix by vortexing for 3 seconds.

5. Add 350 µl 100% isopropanol to the samples and mix by vortexing for 3 seconds.

IMPORTANT: Steps 4. and 5. must be done sequentially, with thorough mixing by vortexing after each addition.

NOTE: For downstream NGS applications a lower concentration of isopropanol may be used to achieve higher DV200 scores [2]. Conversely, for maximum RNA yield with the lower DV200 scores, use a larger volume of isopropanol. See Appendix D for more details.

6. Transfer 600 µl of sample to the RNA Purification Column.

NOTE: Small amounts of residual wax will not interfere with the column purification.

7. Centrifuge the Column/Collection Tube assembly at 11,000 x g for 30 seconds.

8. Discard the flow-through and place the Column back into the Collection Tube.

9. Repeat steps 6 through 8 until all sample has passed through the Column.

10. **1st wash**:
    a. Add 650 µl of prepared RNA Wash Buffer to the RNA Purification Column.
    b. Centrifuge the Column/Collection Tube assembly at 11,000 x g for 30 seconds.
    c. Discard the flow-through and place the Column back into the Collection Tube.

Optional DNA removal step:

The truXTRAC FFPE total NA Plus Kit protocol isolates total RNA that may contain trace amounts of genomic DNA. If DNA-free RNA must be isolated, an optional DNase treatment can be performed.

Note: This optional on-column DNase digestion must be performed after step 10.

See Appendix C for step-by-step instructions.

11. **2nd wash and drying Column**:

    a. Add 650 µl of prepared RNA Wash Buffer to the RNA Purification Column.

    b. Centrifuge the Column/Collection Tube assembly at 16,000 x g for 1 minute.

12. **RNA elution**:

    a. Place the Column into a new RNA Elution Tube (1.5 ml) and add 30 µl (for high concentration) or 50 µl (for high yield) RNA elution buffer to the center of the Column.

IMPORTANT: Even distribution of sample across the column binding matrix is important to get consistent yields. If necessary, tap the column lightly to ensure even distribution of RNA Elution Buffer across the matrix surface.

    b. Incubate for 2 minutes at room temperature.

    c. Spin the Column/Collection Tube assembly at 16,000 x g for 1 minute.

13. Keep the eluted RNA on ice for further processing.

Isolated RNA should be kept at -80˚C for long term storage.

7 – DNA PURIFICATION

1. Set-up the Focused-ultrasonicator following the respective guidelines in Section-4. Use **truXTRAC FFPE microTUBE-500 Tissue Resuspension Time** settings.

2. Set-up the dry-heat blocks as explained in Section-3 and verify the block temperatures to be 56°C. Insert the required number of microTUBE-500 Centrifuge and Heat Block Adapters into the heat block.
   Note: it is recommended to set-up another the dry block heater for a target temperature of 80°C at the same time, which will be necessary for the de-crosslinking steps.

3. Aliquot 110 µl of BE Buffer per sample to be processed into a 1.5 ml microcentrifuge tube. Pre-heat to 80°C. If processing more than one sample, multiply the aliquoted volume by the number of samples. Continue to keep the buffer at 80°C until needed for elution of DNA from column.

4. Prepare Total NA Plus Tissue Lysis Buffer/Proteinase K Mix DNA in a microcentrifuge tube following instructions in Table 2 and mix by inverting 10 times or vortexing for 3 seconds.

NOTE: The Total NA Plus Tissue Lysis Buffer/Proteinase K Mix should be stored at room temperature. Use within 30 min after preparation.

Table 2 – Total NA Plus Tissue Lysis Buffer/Proteinase K Mix for DNA

Reagent	Volume for one sample*	Volume for N samples*
Tissue Lysis Buffer	**352** µl	**352** µl x N
Proteinase K Solution	**88** µl	**88** µl x N

* calculation includes 10% excess in final volume

5. Open the microTUBE with the DNA-containing tissue pellet and add 400 µl of the Total NA Plus Tissue Lysis Buffer/Proteinase K Mix for DNA. Re-cap the Screw-Cap microTUBE tightly.

6. Process the sample in the Focused-ultrasonicator, using **truXTRAC FFPE microTUBE-500 Tissue Resuspension Time** settings (Section-4).

7. Remove the microTUBE-500 from the Focused-ultrasonicator and proceed to Proteinase K treatment:
    a. Load the microTUBE into the microTUBE-500 Centrifuge and Heat Block adapter.
    b. Place into the dry heat block and incubate for 60 minutes at 56°C.
    c. Remove tube together with the microTUBE-500 adapter from the heat block and transfer directly to dry heat block set-up for 80°C incubation.

NOTE: The Proteinase K-treated sample can be stored at room temperature for up to an additional hour. Do not chill on ice.

NOTE: For 10 µm scrolls, tissuePICK and sectionPICK samples 60 minutes Proteinase K incubation time is sufficient. If using 15 µm scrolls or 15 gauge cores it is recommended to increase the incubation time to 2 hours or up to overnight.

8. De-crosslinking:
    a. Incubate for 60 minutes at 80°C.
    b. Remove from the heat block and let cool for 3 minutes at room temperature.

NOTE: Do not chill on ice.

9. Transfer the entire sample to a clean 2 ml microcentrifuge tube.

Optional RNA removal step:

At this point the sample can be treated with RNase A to remove residual RNA before continuing with DNA purification.

Add 5µl of RNase A solution and incubate for 5 minutes at room temperature then continue to step 10.

10. Add 560 µl Total NA Plus B1 Buffer to the sample and vortex for 3 seconds.

11. Add 640 µl 200 Proof ethanol to the sample and vortex for 3 seconds.

IMPORTANT: Steps 10. and 11. must be done sequentially, with thorough mixing after each addition.

12. Place a DNA Purification Column into a Collection Tube.

13. Transfer 600 µl of sample to the DNA Purification Column.

14. Spin the Column/Collection Tube assembly at 11,000 x g for 1 minute.

15. Discard the flow-through and place the Column back into the Collection Tube.

16. Repeat steps 13 through 15 until all sample has passed through the Column.

17. **1st wash**:
    a. Add 500 µl BW Buffer to the DNA Purification Column.
    b. Spin the assembly at 11,000 x g for 1 minute.
    c. Discard the flow-through and place the Column back into the Collection Tube.

18. **2nd wash**:
    a. Add 600 µl of B5 Buffer to the DNA Purification Column.
    b. Spin the assembly at 11,000 x g for 1 minute.
    c. Discard the flow-through and place the Column back into the Collection Tube.

19. **Dry Column**: Spin the assembly at 16,000 x g for 1 minute.

20. **Elute DNA - 1st step**:
    a. Place the Purification Column into a clean 1.5 ml microcentrifuge tube.
    b. Add 50 µl of pre-warmed BE Buffer (80 °C) to the center of the Column.
    c. Leave Column at room temperature for 3 minutes.
    d. Spin the Column/microcentrifuge tube assembly at 11,000 x g for 1 minute.

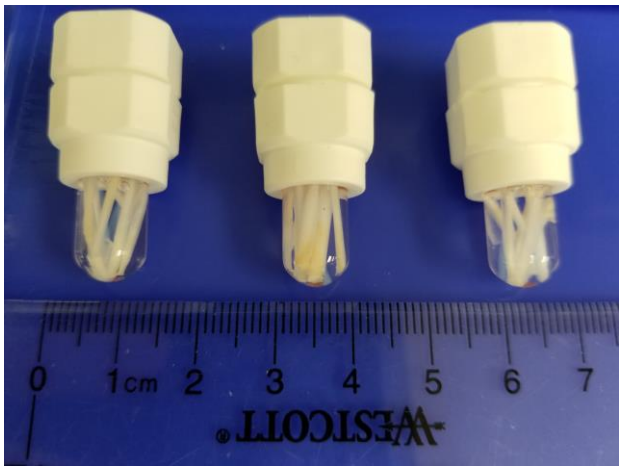
IMPORTANT: Even distribution of sample across theis important to get consistent yields. If necessary, tap the column lightly to ensure even distribution of Buffer BE across the matrix surface.

21. **Elute DNA – 2nd step**:
    a. Add a second 50 µl aliquot of pre-warmed BE Buffer (80 °C) to the center of the Column.
    b. Incubate again at room temperature for 3 minutes.
    c. Spin the Column/microcentrifuge tube assembly at 11,000 x g for 1 minute.

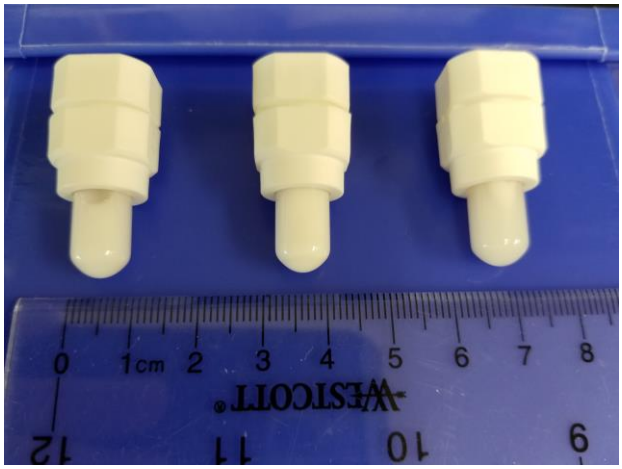
APPENDIX A – EXAMPLE OF AFA-BASED PARAFFIN EMULSIFICATION AND TISSUE REHYDRATION

The picture below represents how the FFPE tissue appears before (top) and after (bottom) **truXTRAC FFPE microTUBE-500 Emulsification** treatment.

The paraffin in the FFPE tissue (here four 10 µm kidney tissue sections) was emulsified in microTUBE-500 Screw-Caps using a Covaris LE220 Focused-ultrasonicator.



Before AFA



After AFA

APPENDIX B – TROUBLESHOOTING GUIDE

Issue	Cause	Solution	Comments / Suggestions
Low yield of RNA and/or DNA	Low tissue to wax ratio in FFPE section.	Trim off any excess paraffin before sectioning a FFPE tissue block. Repeat the procedure using additional sections until desired yield is achieved.	In your initial use of the truXTRAC FFPE total NA Plus kit use FFPE blocks that have been well characterized for yield and quality.
	Insufficient tissue input.	Select FFPE section with higher tissue to wax ratio or add additional section	See sample input guidelines in Section-1
	PK in solution denatured or expired.	Repeat the procedure using fresh PK solution.	Always store PK solution as recommended.
No RNA yield	Ethanol not added to RNA Wash Buffer.	Repeat the procedure with fresh samples and ensure ethanol is added to RNA Wash Buffer.	
RNA concentration is low	Elution volume is too high.	Repeat procedure using a lower elution volume (30 µl minimum volume is required). Alternatively, concentrate samples using ethanol precipitation or other suitable volume reduction methods.	
	Steps 4. And 5. In Section-6 were not done correctly.	Make sure B1 Buffer and 100% Isopropanol are added sequentially. Mix well after each addition.	
No or low DNA yield	Ethanol not added to B5 Buffer.	Repeat the procedure with fresh samples and ensure ethanol is added to B5 Buffer.	
	Parts or entire tissue pellet lost during supernatant removal.	Repeat using narrow mouth 200 µl pipette tip to take off RNA-containing supernatant.	Follow guidelines in the protocol closely. Make sure laboratory personnel is trained in procedure.
DNA concentration is too low	Elution volume is too high.	Repeat procedure using a lower elution volume (50 µl minimum volume is required). Alternatively, concentrate samples using ethanol precipitation or other suitable volume reduction methods.	
DNA does not perform well in downstream applications such as qPCR	DNA in FFPE sample blocks is severely cross-linked or degraded.	Design amplicons to be as small as possible (<100 bp).	DNA isolated using Covaris AFA technology is of the highest possible quality. Some FFPE sample blocks may be too degraded or cross-linked for some applications.

APPENDIX C – OPTIONAL DNASE TREATMENT OF EXTRACTED RNA

The truXTRAC FFPE total NA kit isolates total RNA that may contain small amounts of DNA. An optional DNase treatment protocol is provided if DNA-free RNA is desired.

It is recommended to use the TURBO DNA-*free* kit (ThermoFisher PN AM1907).

1. Place the RNA Columns into new collection or 2.0 ml microcentrifuge tubes after the 1st wash Step 10 in Section-6 (RNA Purification).

2. Prepare a 1 X TURBO DNase mixture directly on the RNA Purification column by adding:

   · RNase-free H_2O 88 µl
   · 10X TURBO DNase buffer 10 µl
   · TURBO DNase 2 µl

3. Close cap and vortex to mix.

4. Incubate at room temperature for 30 minutes.

5. Sequentially add 175 µl B1 Buffer and then 300 µl 65% isopropanol.

6. Close cap and vortex to mix.

7. Spin at 11, 000 x g for 30 seconds.

8. Pipette the flow-through in the collection tube back into the Column.

IMPORTANT: Do not discard the flow through. It contains RNA!

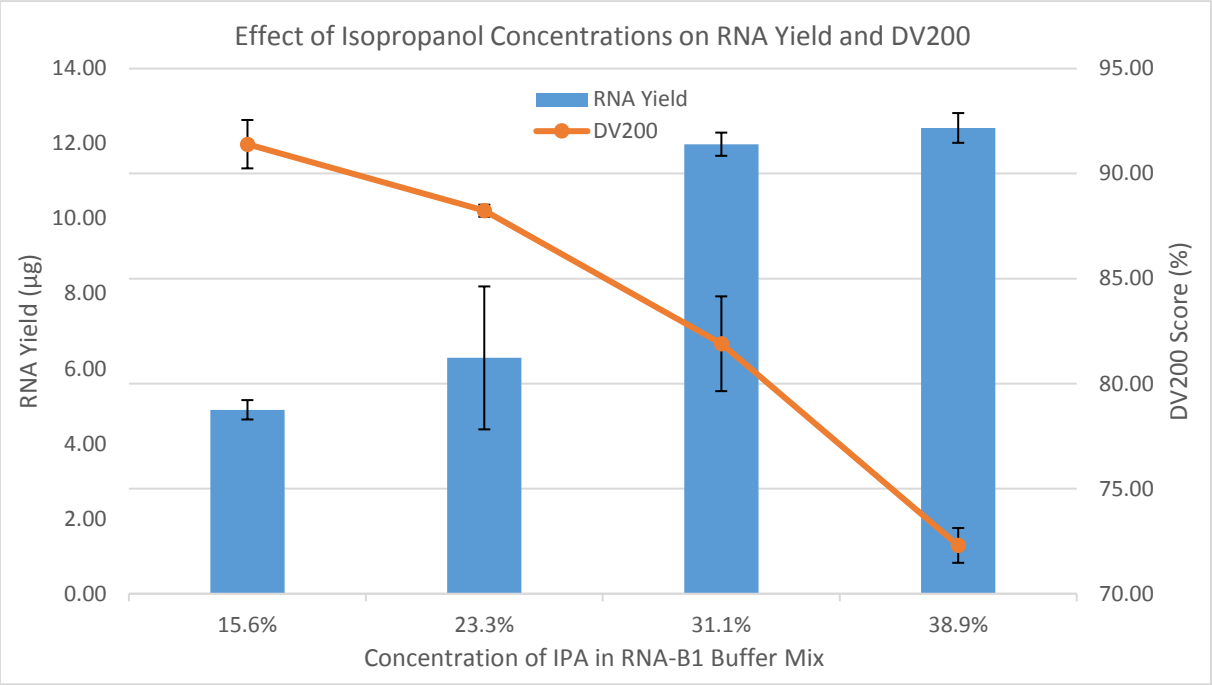
9. Spin at 11, 000 x g for 30 seconds.

10. Now, discard the flow-through and place the Purification Column back onto the Collection Tube.

11. Proceed with Step 11 in Section-6 (RNA Purification).

APPENDIX D – ISOPROPANOL CONCENTRATION AND DV200 SCORES

The isopropanol concentration used in the Section 3 - RNA purification will impact RNA yield and size distribution (as expressed by DV200 score [2]). If high DV200 scores are desirable, use less concentrated isopropanol. However, if maximum RNA yield is desired at the expense of the DV200 score (increase of <200nts RNA fraction), use a larger volume of 100% isopropanol. The effects on FFPE RNA yield and DV200 score can be seen in the Figure below.



Final IPA (%)	Stock IPA (%)	IPA Volume (µl)	B1 Buffer Volume (µl)	RNA Volume (µl)
15.6	50	350	375	400
23.3	75	350	375	400
31.1	100	350	375	400
38.9	100	560	480	400

Changing the final volume of the RNA-Isopropanol mixture will also change column binding conditions, requiring the addition of extra B1 Buffer. Refer to the following table to calculate appropriate volumes of isopropanol and B1 Buffer required when using more than 350 µl 100% IPA.

Additional 100% Isopropanol	Additional B1 Buffer
X µl	0.5X µl

For example, when adding an extra 100 µl 100% isopropanol (450 µl isopropanol total), you must also add another 50 µl Total NA Plus B1 buffer (425 µl Total NA Plus B1 buffer total).

APPENDIX

To calculate the necessary volume of 100% isopropanol beyond 350 µl (X) to achieve a specific final isopropanol concentration in the RNA-B1 Buffer mix (Y), use the following equation:

$$X = \frac{35000 - 1125Y}{1.5Y - 100}$$

Alternatively, to calculate the final concentration of isopropanol in the RNA-B1 Buffer mix (Y) when using a known volume of 100% isopropanol in excess of the default 350 µl (X), use this equation:

$$Y = \frac{35000 + 100X}{1125 + 1.5X}$$

TIPS FOR DETERMINING QUALITY AND QUANTITY OF THE PURIFIED FFPE DNA/RNA

- To determine DNA and RNA yields, a fluorometric assay such as Qubit ™ (Life Technologies) should be used.
- In addition, spectrophotometric analysis determining the A260/280 and A260/230 ratios will determine if protein or peptide/salt contamination is present in the sample.
- qPCR can be used to assess the amplifiability of isolated DNA as well as the presence of inhibitors. Note that DNA from FFPE tissue itself can act as inhibitor at high input concentrations due to the extensive damage (nicks, depurination, etc.). Therefore, a dilution series over at least 5 orders of magnitude starting with undiluted material of the extracted DNA should always be done when assessing quality by qPCR. An example is shown in Dietrich et al. Figure 1 [3].

ADDITIONAL NOTES

1. See following link: http://covaris.com/resources/protocols/ for updates to this document.
2. The treatment settings listed in this document are recommended guidelines. Actual results may vary depending on the tissue type, mass, and previous handling of FFPE samples.
3. Covered by US Patent 9,080,167
4. Other patents pending

REFERENCES

1. Carrick et al. (2015) Robustness of Next Generation Sequencing on Older Formalin-Fixed Paraffin-Embedded Tissue. PLoS ONE 10(7): e0127353.
2. Landolt et al. (2016) RNA extraction for RNA sequencing of archival renal tissues. Scand J Clin Lab Invest 76(5):426-434.
3. Dietrich et al. (2013) Improved PCR Performance Using Template DNA from Formalin-Fixed and Paraffin-Embedded Tissues by Overcoming PCR Inhibition. PLOS one 8(10):e77771