truXTRAC® FFPE
total Nucleic Acid Plus Kit - Column

Adaptive Focused Acoustics® (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 sequential 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, while simultaneously rehydrating the tissue. Compared to traditional passive, chemical-based methods of paraffin removal, this non-contact mechanical process is more efficient as the paraffin is removed and emulsified from the tissue. Uniquely, AFA enables increased yields of nucleic acids and minimizes the degradation of nucleic acids exposed at the FFPE section surface. The truXTRAC protocol results in high yields of high-quality RNA and DNA for sensitive analytical methods such as next-generation sequencing (NGS) or qPCR/RT-qPCR.

This protocol is optimized for FFPE sections for up to a combined thickness of 40 µm. 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 is 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.

More importantly, however, 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. Thus, an advanced mechanical deparaffinization process is important to extract the high quality nucleic acids required for sensitive analytical techniques. Covaris AFA enables non-contact mechanical removal of paraffin from FFPE samples to improve the yield and quality of extracted nucleic acids.

If you require any assistance with this product please refer to Troubleshooting (Appendix A) in this protocol, 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	17MAY2018	New protocol for truXTRAC FFPE Total NA Plus Kit - Column (Curls/Scrolls)
010435	B	7JUNE2018	LE220 water level updated

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 columns
○ DNA Purification Columns	25 columns
○ Collection Tubes	50 tubes
○ RNA Elution Tubes	25 tubes
○ microTUBE-500 AFA Fiber Screw-Cap FFPE	25 tubes

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

STORAGE

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

Store all other kit components at room temperature.

LABORATORY EQUIPMENT, CHEMICALS AND CONSUMABLES SUPPLIED BY USER

Required Laboratory Equipment and Accessories

- microTUBE-500 Centrifuge and Heat Block Adapter (Covaris PN 500503)
- Microcentrifuge (1.5 ml or 2 ml tube compatible, 16,000 x g capability)
- Dry block heater or temperature-controlled water bath able to accurately heat between 50-90C

Required Chemicals and Enzymes

- 100% isopropanol, ultrapure (e.g., AmericanBio, PN AB07015)
- 100% ethanol, molecular biology grade (e.g., AmericanBio, PN AB00515)
- Nuclease-free water (e.g., Ambion, PN AM9930)

Optional Enzymes

- DNase TURBO DNA-free kit (ThermoFisher Scientific PN AM1907)
- Optional DNase-free RNase A (10 mg/ml) (e.g., ThermoFisher Scientific, PN EN0531)

Required 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)

Covaris Focused-ultrasonicator Accessories and Plate Definitions

The table below contains the parts and plate definitions necessary to run the protocol.

Use the parts and plate definitions specific to your Covaris Focused-ultrasonicator.

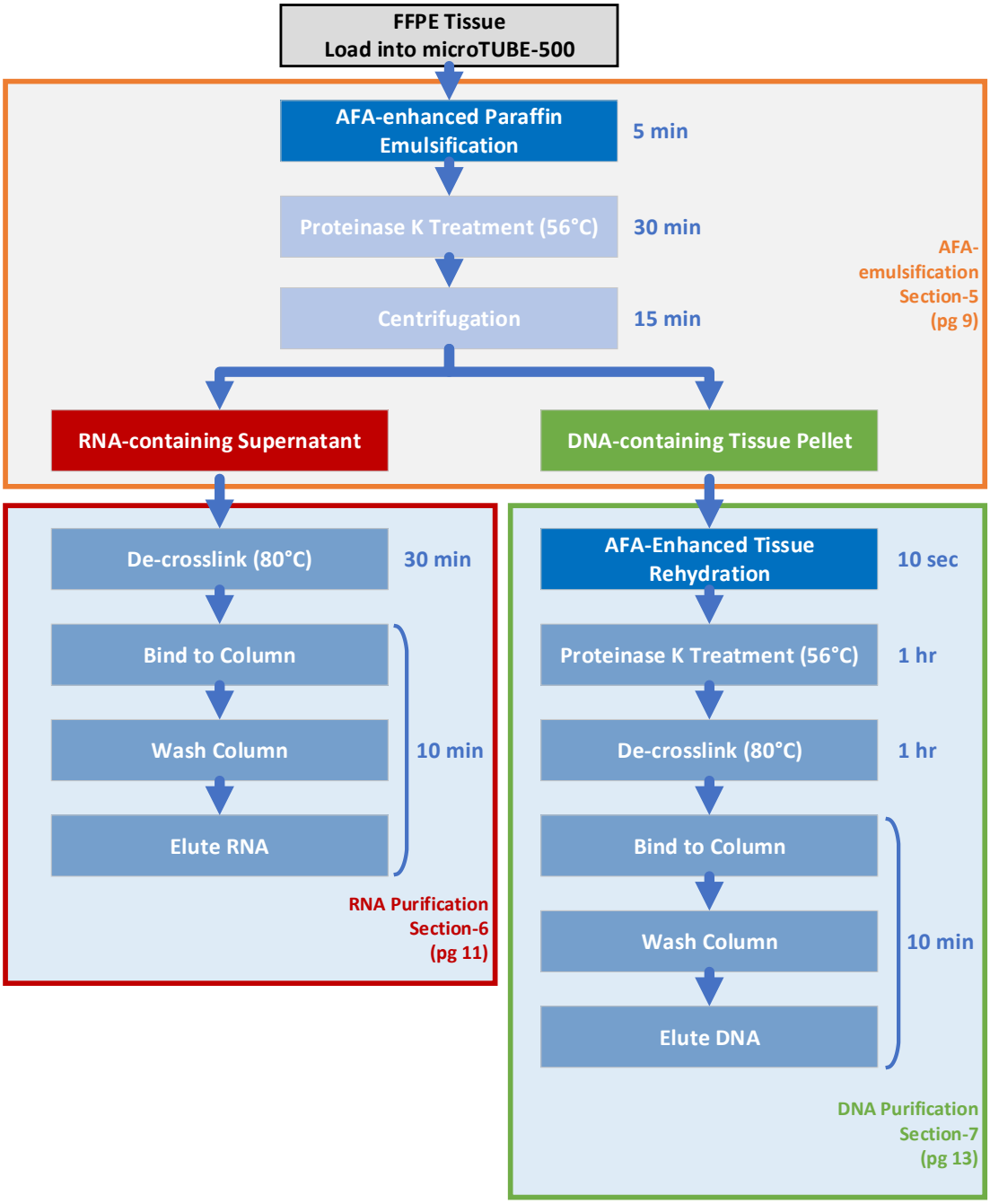
Instrument	M220	ME220	S220	E220 Evolution	E220	LE220 / LE220-plus
Holder/Rack Description (PN)	Holder XTU (500414)	Rack 4 Position microTUBE-500 (500525)	Holder microTUBE-500 Screw Cap (500449)	Rack E220e 4 microTUBE-500 Screw Cap (500484)	Rack 24 microTUBE-500 Screw Cap (500452)	Rack 24 microTUBE-500 Screw Cap (500452)
Plate definition file name	NA	<4 microTUBE-500 Screw-Cap PN 520185>	NA	<500484 E220e 4 microTUBE-500 Screw-Cap>	<500452 Rack 24 Place microTUBE-500 Screw-Cap>	<500452 Rack 24 Place microTUBE-500 Screw-Cap>
Required Accessories (PN)	Insert XTU (500471)	ME220 Waveguide 4 Place (500534)	NA	Intensifier (500141)	Intensifier (500141)	NA

FFPE tNA Extraction And Purification Workflow

Using the Adaptive Focused Acoustics (AFA) process, FFPE tissue section specimens are prepared in Total NA Plus Tissue Lysis Buffer in the presence of Proteinase K, followed by an incubation at 56C for a short duration. This results in the release of RNA while minimizing 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.

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



1 – FFPE Sample Input Requirements and Guidelines – Curls/Scrolls

The truXTRAC protocol is highly efficient at mechanically removing paraffin, while simultaneously rehydrating the tissue.

Curls/scrolls input requirements:

The maximum input for scrolls/curls per microTUBE-500 is a total thickness of 40 µm. For example, 2 scrolls at 10 µm thick equals a total thickness of 20 µm.

Refer to the table below for input requirements for standard scroll/curl thickness

FFPE Curls/Scrolls Thickness	Maximum Scrolls per microTUBE
5 µm	**6**
10 µm	**3**
15 µm	**2**

CAUTION: Do NOT exceed the amount of FFPE material provided in the table above. Overloading will negatively impact the quality and quantity of extractable nucleic acids.

2 – Preparation of Reagents

Follow these instructions before starting the FFPE tNA isolation protocol.

1. **RNA Wash Buffer:** Before initial use, add 40 ml of 100% 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**: Before initial use, add 28 ml of 100% 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 for a white precipitate that may form during storage. If white precipitate is visible, incubate the buffer bottles at 50 – 60C for 5 to 10 minutes before use to dissolve any precipitate.

3 – Preparation of Heat Blocks

1. Preheat dry block heaters to 56C and 80C ± 3C. It is critical that these temperatures are accurate in order to successfully execute the protocol.

2. Test the temperature of your water bath and heat blocks:
   a. Place a microcentrifuge tube (1.5 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 56C or 80C ± 3C.

CAUTION: The Covaris microTUBE must be used in conjunction with Covaris Centrifuge and Heat Block microTUBE Adapters (PN 500503).

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

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 see a Plate Definition on your system, please contact Covaris Technical Support (techsupport@covaris.com)

Refer to page 4 for Plate Definitions and required Focused-ultrasonicator accessories

1. **Create "Acoustic Paraffin Emulsification" program in SonoLab™**
   Use the settings provided in the table below, specific to your Covaris instrument type, to create a program called "Acoustic Paraffin Emulsification" using the Covaris SonoLab method editor. Save the program for later use.

Instrument	M220	ME220	S220	E220 Evolution	E220	LE220
Peak Incident Power (PIP) (Watt)	75	75	200	200	200	450
Duty Factor (%)	20	25	30	30	30	30
Cycles Per Burst (CPB)	200	1000	200	200	200	200
Treatment time (seconds)	300	300	300	300	300	300
Bath temperature (°C)	20	20	20	20	20	20
Water Level (run)	Full	Auto	8	8	8	8

2. **Create "Acoustic Pellet Resuspension" program in SonoLab™**
   Use the settings provided in the table below, specific to your Covaris instrument type, to create a program called "Acoustic Pellet Resuspension" using the Covaris SonoLab method editor. Save the program for later use.

Instrument	M220	ME220	S220	E220 Evolution	E220	LE220
Peak Incident Power (PIP) (Watt)	75	75	200	200	200	450
Duty Factor (%)	20	25	30	30	30	30
Cycles Per Burst (CPB)	200	1000	200	200	200	200
Treatment time (seconds)	10	10	10	10	10	10
Bath temperature (°C)	20	20	20	20	20	20
Water Level (run)	Full	Auto	8	8	8	8

5 – Paraffin Emulsification & Tissue Rehydration

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

> ⚠ CAUTION: The Total NA Plus Tissue Lysis Buffer/Proteinase K Mix should be stored at room temperature and used 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

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

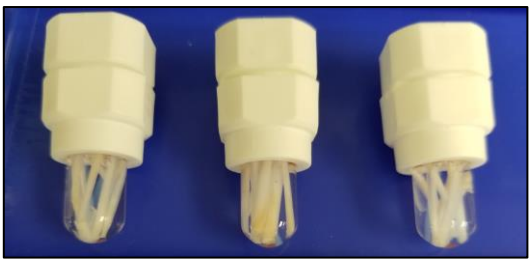
3. Add 440 µl Tissue Lysis Buffer/Proteinase K mix to the microTUBE-500.

4. Close the microTUBE tightly with the Screw-Cap and transfer the microTUBE-500 to the appropriate rack or holder/insert for your Focused-ultrasonicator. Load the rack or holder/insert containing the microTUBE(s) into the Focused-ultrasonicator for processing.

5. Process the sample using the "***Acoustic Paraffin Emulsification***" program on the Focused-ultrasonicator.

   Note: It is expected that the solution will turn milky white. See example below.

Before



After

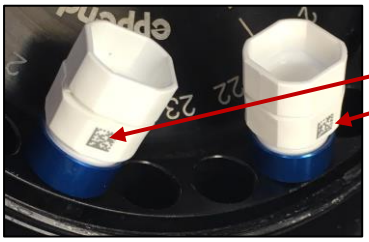
6. Remove the microTUBE-500 from the Focused-ultrasonicator and load it into the microTUBE-500 Centrifuge and Heat Block adapter

   When processing in batches, samples may be kept at room temperature for up to two hours prior to Proteinase K incubation at 56C (Step 7).

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

CAUTION: Do not chill on ice as rapid cooling will cause detergents to precipitate.

8. 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.

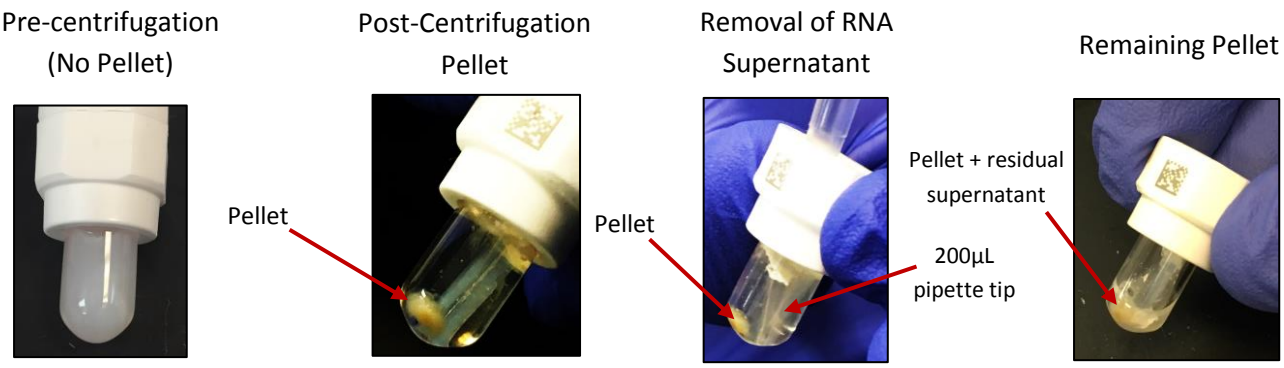


Note: The centrifuge lid may not close and may need to be left off during centrifugation.

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

CAUTION: This is a critical step in the workflow. By following these guidelines, the risk of losing the DNA-containing tissue pellet 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. The pellet may appear faint and difficult to see.
b. Tilt the tube slightly away from the pellet.
c. Using a 200 µl pipette with a 200 µl pipette tip, slowly pierce the upper emulsified wax layer 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 pellet and barcode. DO NOT USE WIDE-MOUTH TIPS.
d. A layer of emulsified wax may descend obscuring the pellet. This is normal. Leave 20-30 µl of supernatant behind. This will not significantly impact RNA or DNA yield.



10. Save the DNA-containing tissue pellet for subsequent DNA purification as described in Section-7. **Proceed immediately to RNA Purification (Section-6).**

The DNA-containing pellet can be stored on ice or at 2–8C for up to 1 day. For longer periods, store at –15 to –30C.

6 - RNA PURIFICATION

1. Set up the dry-heat block as explained in Section-3 and verify the block temperature to be 80C.
2. Incubate the 2 ml microcentrifuge tube with the RNA-containing supernatant at 80C for 20 minutes. Remove tubes and cool at room temperature for 3 minutes.
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.

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

RNA Yields and DV200 Scores:

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 C 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 at room temperature. All subsequent centrifugations are performed at room temperature.
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 is required for downstream applications such as RNA-seq, an optional DNase treatment may be performed to remove DNA.

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

See Appendix B 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.

CAUTION: 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. Centrifuge the Column/RNA Elution Tube assembly at 16,000 x g for 1 minute Incubate for 2 minutes at room temperature.

    d. Remove the Column from the RNA Elution Tube and save the RNA Elution Tube containing the eluted RNA.

For high RNA yield and concentration, the RNA eluate from Step 12d may be reapplied to the column and steps 12b through 12d repeated.

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

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

7 – DNA PURIFICATION

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

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

CAUTION: The Total NA Plus Tissue Lysis Buffer/Proteinase K Mix should be stored at room temperature and used 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

3. 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.

4. Transfer the sample into your Covaris Focused-ultrasonicator.

5. Process the sample using the "***Acoustic Pellet Resuspension***" program on your Covaris Focused-ultrasonicator.

6. Remove the microTUBE-500 from the Focused-ultrasonicator and load the microTUBE into the microTUBE-500 Centrifuge and Heat Block adapter.

7. Incubate for a minimum of 60 minutes at 56C.

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

CAUTION: It is recommended to increase the incubation time to 2 hours or up to overnight for curls/scrolls ≥ 15 µm thickness.

8. Remove microTUBE-500 together with the microTUBE-500 adapter from the heat block and transfer directly to dry heat block set-up for 80C incubation. Incubate for 60 minutes at 80C.

9. Remove microTUBE-500 with adapter from the heat block and let cool for 3 minutes at room temperature.

10. 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 5 µl of RNase A (10 mg/ml) solution and incubate for 5 minutes at room temperature, then continue to step 11.

CAUTION: Steps 11 and 12 must be done sequentially, with thorough mixing by vortexing after each addition.

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

12. Add 640 µl 100% ethanol to the sample and vortex for 3 seconds.

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

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

15. Centrifuge the Column/Collection Tube assembly at 11,000 x g for 1 minute at room temperature. All subsequent centrifugations are performed at room temperature.

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

17. Repeat steps 14 through 16 until all sample has passed through the Column.

18. **1st wash**:
    a. Add 500 µl BW Buffer to the DNA Purification Column.
    b. Centrifuge 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. **2nd wash**:
    a. Add 600 µl of B5 Buffer to the DNA Purification Column.
    b. Centrifuge the assembly at 11,000 x g for 1 minute.
    c. Discard the flow-through and place the Column back into the Collection Tube.

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

21. **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 (80C, from Step 1) to the center of the Column.

c. Incubate at room temperature for 3 minutes.

d. Centrifuge the Column/microcentrifuge tube assembly at 11,000 x g for 1 minute.

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

22. **Elute DNA – 2nd step**:

a. Add a second 50 µl aliquot of pre-warmed BE Buffer (80C) to the center of the Column.

b. Incubate at room temperature for 3 minutes.

c. Centrifuge the Column/microcentrifuge tube assembly at 11,000 x g for 1 minute.

d. Remove the Column from the microcentrifuge tube and save the microcentrifuge containing the eluted DNA.

For high DNA yield and concentration, the DNA eluate from Step 22d may be reapplied to the column and steps 22b through 22d repeated.

APPENDIX A – 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.
	PK digestion time insufficient	Increase incubation times to 2 hours or up to overnight for curls/scrolls sections ≥ 15 µm thickness.	Curls/scrolls sections ≥ 15 µm thickness may require greater PK incubation time
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 100% 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 B – 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.

The protocol below describes removal of DNA specifically using TURBO DNA-*free* kit (ThermoFisher Scientific 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 master mix for N samples with 10% excess volume:

   · RNase-free H_2O 88 µl x N x 1.1
   · 10X TURBO DNase buffer 10 µl x N x 1.1
   · TURBO DNase 2 µl x N x 1.1

3. Close cap and invert gently to mix.

4. Add 100 µl of the DNase master mix to the column and incubate at room temperature for 30 minutes.

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

6. Close cap and vortex to mix.

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

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

CAUTION: Do not discard the flow-through as it contains the RNA

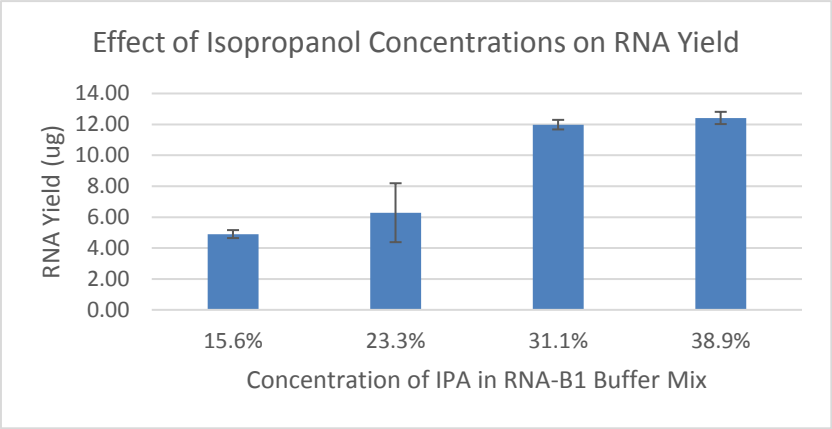
9. Centrifuge at 11,000 x g for 30 seconds.

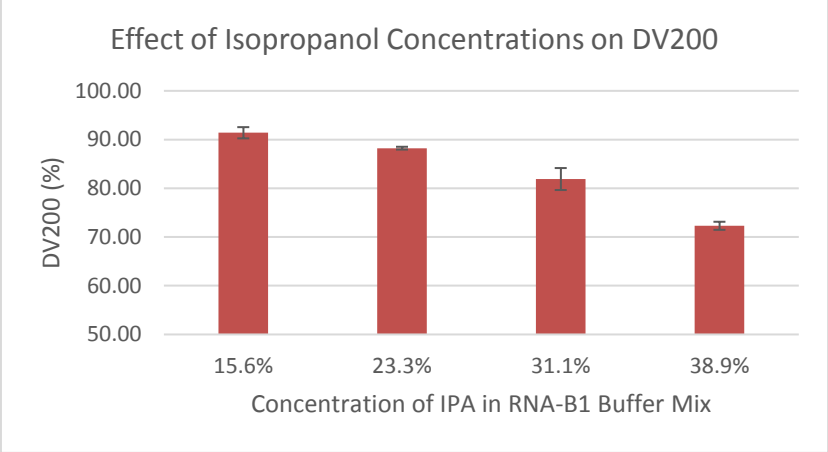
10. Discard the flow-through and place the Purification Column back onto the Collection Tube.

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

APPENDIX C – ISOPROPANOL CONCENTRATION AND DV200 SCORES

The isopropanol concentration used in 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 Figures 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

APPENDIX

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% isopropanol.

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).

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 an inhibitor at high input concentrations due to the extensive damage (e.g., nicks and/or depurination). 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 and issued

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