Covaris®

truXTRAC® FFPE total NA (tNA) Ultra Kit - Column

Adaptive Focused Acoustics® (AFA®)-based Sequential RNA and DNA Extraction from FFPE Tissues using Column-based Purification

PN 520307

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Intended Use

The truXTRAC FFPE total NA (tNA) Ultra Kit - Column (PN 520307) 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 (tNA) Ultra Kit - Column 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) and column-based purification.

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 at paraffin removal and emulsification from the tissue. Uniquely, AFA enables increased yields of nucleic acids and minimizing 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 up to 20 µm of total FFPE tissue sections. For sample inputs that differ from the requirements or for very small inputs, please contact ApplicationSupport@covaris.com.

Important Notes on FFPE Samples:

The yield of DNA and RNA from FFPE tissue blocks is highly variable. Factors such as fixation time, ratio of tissue to paraffin, type of tissue, and age and storage conditions of the FFPE block are the main causes for variability in yields.

More importantly, the quality of DNA and RNA isolated from FFPE samples can 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 higher 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 [2].

Note for users:

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
010534	A	8/2020	Initial release of truXTRAC FFPE total NA Ultra Kit - Column
010534	B	3/2021	Update sample input requirements and correct typographical errors.

Kit Contents

- 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
- Buffer BE ..... 7.5 ml
- RNA Purification Columns 25
- DNA Purification Columns 25
- Collection Tubes 50
- RNA Elution Tubes 25
- AFA-TUBE PP Screw-Cap 0.5 ml 25

SDS Information available at: www.covaris.com/safety-data-sheets/

Storage

Upon kit arrival, store the Proteinase K solution at 2 °C to 8 °C. Store all other kit components at ambient temperature.

Laboratory Equipment, Chemicals, and Consumables Supplied by User

Required Laboratory Equipment and Accessories

- Microcentrifuge with fixed angle rotor for 2 ml tubes (16,000 x g capability)
- 0.5 ml Centrifuge Adapters (Eppendorf, PN 022636227)
- Dry block heater with blocks to accommodate 2 ml tubes or temperature-controlled water bath able to accurately heat between 50 °C to 90 °C
- Dry block heater with blocks to accommodate 0.5 ml microcentrifuge conical tubes or temperature-controlled water bath able to accurately heat between 50 °C to 90 °C

Required Chemical and Enzymes

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

Optional Enzymes

- TURBO DNase (2 U/µL) (Thermo Fisher Scientific, PN AM2238), containing 10X TURBO DNase Buffer
- DNase-free RNase A (10 mg/ml) (e.g., Thermo Fisher Scientific, PN EN0531)

Required Consumable

- Eppendorf tubes 2 ml (Eppendorf, PN 0022363344)
- Tubes to make buffer mixtures

Covaris Focused-ultrasonicator Accessories and Plate Definitions

The table below contains the accessories and plate definitions necessary to run the protocol. Use the parts and plate definitions specific to your Covaris Focused-ultrasonicator.

Instrument	M220	ME220
Holder/Rack Description (PN)	Holder XTU (PN 500414)	ME220 Rack 4 Place AFA-TUBE PP Screw-Cap 0.5 ml 500700)*
Plate Definition File Name	N/A	<500700 Rack 4 Place AFA-TUBE PP Screw-Cap 0.5 ml>*
Required Accessories (PN)	Insert XTU (PN 500692)	Wave Guide 8 Place (500526)

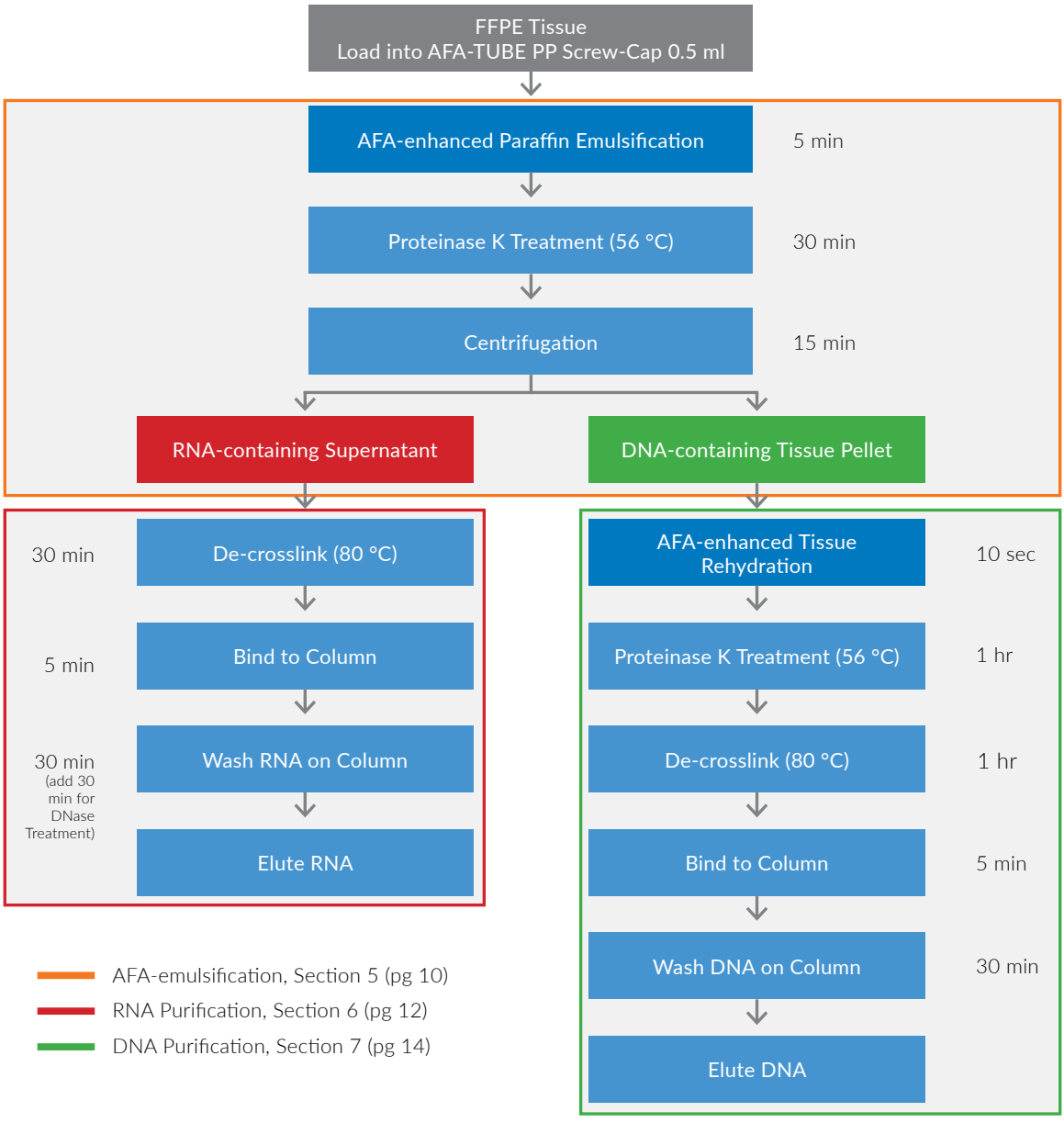
* Requires SonoLab 8.0.2.1017 or Higher. Please contact ApplicationSupport@covaris.com.

FFPE tNA Extraction and Purification Workflow

Using the Adaptive Focused Acoustics (AFA) process, FFPE samples are prepared in Tissue Lysis Buffer in the presence of Proteinase K, followed by an incubation at 56 °C 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 at 80 °C. DNA is then purified over a spin column.



1 - FFPE Sample Input Requirements and Guidelines

> **CAUTION**: Do NOT exceed the input requirements in the tables below. Overloading will negatively impact the quality and quantity of extractable nucleic acids.

Slide Section Input Requirements:

Slide Collection Method	Maximum Input per AFA-TUBE PP Screw-Cap 0.5 ml
Scalpel or razor blade to scrape material from slides	20 µm of total thickness (e.g., 4 slides at 5 µm thick = 20 µm total thickness) Max Area (on each slide): 10 mm x 10 mm

Curls/Scrolls Input Requirements:

For best results, minimize the amount of wax present by trimming. No more than 1-part wax to 1-part tissue is recommended.

FFPE Curl/Scroll Thickness	Maximum Scrolls per AFA-TUBE PP Screw-Cap 0.5 ml
5 µm	4
10 µm	2
15 µm	1

For inputs not listed please contact ApplicationSupport@covaris.com.

2 - Preparation of Reagents

> **NOTE**: 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 ambient 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 ambient temperature. Minimize the number of times the bottle is opened to avoid evaporation of ethanol.

3. ***Total NA Plus B1 Buffer and Tissue Lysis Buffer***: Visually check for a white precipitate that may form during storage before each use. If white precipitate is visible, incubate the buffer at 50 °C to 60 °C for 5 to 10 minutes before use to dissolve any precipitate.

3 - Preparation of Heat Blocks

1. Preheat dry block heaters to 56 °C and 80 °C ± 3 °C. It is crucial that these temperatures are accurate to successfully execute the protocol.
2. To test the temperature of your water bath and heat blocks:
    a. Place an AFA-TUBE PP Screw-Cap 0.5 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 AFA-TUBE PP Screw-Cap 0.5 ml has reached 56 °C or 80 °C ± 2 °C.

> **CAUTION**: The AFA-TUBE PP Screw-Cap 0.5 ml must be used in conjunction with a compatible heat block, such as, Eppendorf SmartBlock™ 0.5 mL, thermoblock for 24 reaction vessels 0.5 mL, incl. Transfer Rack 0.5 mL (Eppendorf, Cat No. 5361000031). It is important to use an accurate heating source for incubation of AFA-TUBE PP Screw-Cap 0.5 ml 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).

NOTE: 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 the "Acoustic Paraffin Emulsification" program using the Covaris SonoLab method editor. Save the program for later use.

Instrument	M220	ME220
Peak Incident Power (PIP) (Watt)	50	50
Duty Factor (%)	15	15
Cycles Per Burst (CPB)	800	800
Treatment time (seconds)	300	300
Bath temperature (C)	20	20
Water Level (run)	Full	Automatic

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
Peak Incident Power (PIP) (Watt)	50	50
Duty Factor (%)	15	15
Cycles Per Burst (CPB)	800	800
Treatment time (seconds)	10	10
Bath temperature (C)	20	20
Water Level (run)	Full	Automatic

5 - Paraffin Emulsification, Tissue Rehydration, and Lysis

1. Prepare **Tissue Lysis Buffer/PK Solution Mix** by following the instructions in Table 1 below. Mix by inverting 10 times or vortexing for 3 seconds.

> **CAUTION**: The Tissue Lysis Buffer/PK Solution Mix should be stored at ambient temperature and used within 30 min after preparation.

Reagent	Volume for one sample*	Volume for N samples*
Tissue Lysis Buffer	510 µl	510 µl x N
PK Solution	40 µl	40 µl x N

Table 1. Tissue Lysis Buffer/PK Solution Mix. *Calculation includes 10% excess in final volume.

2. Load each FFPE sample into the AFA-TUBE PP Screw-Cap 0.5 ml.
3. Add **500 µl** of the **Tissue Lysis Buffer/PK Solution Mix** into each AFA-TUBE PP Screw-Cap 0.5 ml. Ensure that the FFPE sample is fully immersed in the buffer.
4. Close the AFA-TUBE PP Screw-Cap 0.5 ml tightly with the Screw-Caps and transfer the AFA-TUBE PP Screw-Cap 0.5 ml to the appropriate rack or holder/insert for your Focused-ultrasonicator.
5. Load the rack or holder/insert containing the AFA-TUBE PP Screw-Cap 0.5 ml into the Focused-ultrasonicator for processing.
6. Process the samples using the “Acoustic Paraffin Emulsification” program.

> **NOTE**: It is expected that the solution will turn milky white or yellow. See example below.

Before AFA

After AFA

7. Transfer the AFA-TUBE PP Screw-Cap 0.5 ml from the Focused-ultrasonicator to the heat block.

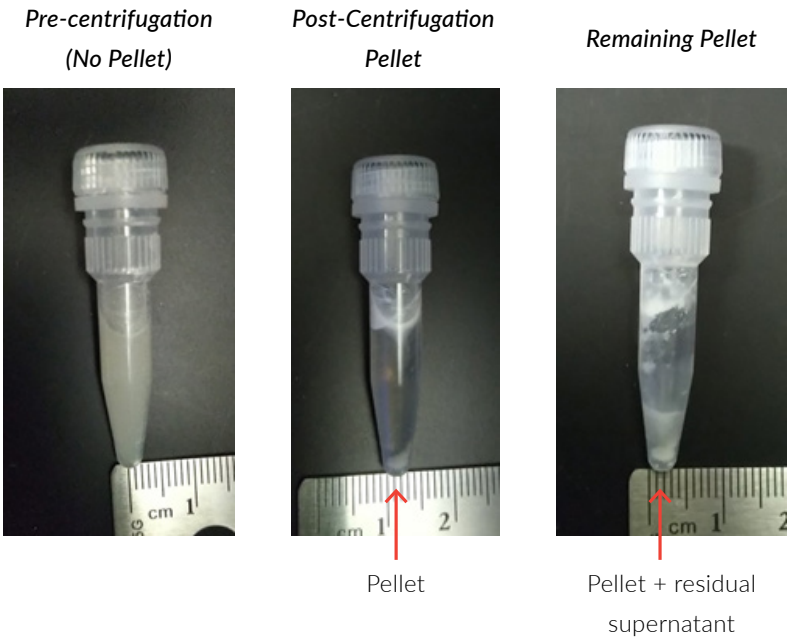
> **NOTE**: When processing in batches, the samples may be kept at ambient temperature for up to two hours prior to PK Solution incubation at 56 °C (Step 8).

8. Incubate all samples for 30 minutes at 56 °C. Remove the AFA-TUBE PP Screw-Cap 0.5 ml from the heat block, and let them cool at ambient temperature for 3 min.
9. Place the AFA-TUBE PP Screw-Cap 0.5 ml in centrifuge adapters. Then transfer AFA-TUBE PP Screw-Cap 0.5 ml into a microcentrifuge (fixed angle rotor) and centrifuge at 5,000 x g for 15 minutes.

> **NOTE**: The internal centrifuge lid may not close and may need to be left off during centrifugation.

10. Remove the AFA-TUBE PP Screw-Cap 0.5 ml from the microcentrifuge, remove Screw-Cap, and carefully transfer 450 µl of the supernatant to 2 ml microcentrifuge tubes.

> **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 at the bottom of the tube with more pellet toward the side faces outward during centrifugation. The pellet may appear small and difficult to see.
> b. Using a **200 µl** pipette tip, slowly and carefully pierce the upper emulsified wax layer and remove 200 µl of supernatant while simultaneously lowering the tip following the liquid level. Place the pipette tip 2 to 3 mm above the pellet, towards the tube wall that faces away from the pellet. Use the same 200 µl pipette for a total of three times to remove the remaining supernatant.
> **DO NOT USE WIDE-MOUTH PIPETTE TIPS**.
> c. A layer of emulsified wax may descend obscuring the pellet. This is normal. Leave **approximately 50 µl** of supernatant behind. This will not significantly impact RNA or DNA yield.



> **NOTE**: If the pellet becomes dislodged from the bottom of the AFA-TUBE PP Screw-Cap 0.5 ml before the RNA supernatant has been removed, repeat centrifugation (Step 9) to re-form pellet. Remove RNA supernatant as described in Step 10.

11. 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 °C to 8 °C for up to 1 day. For longer periods, store between −15 °C and −30 °C.

6 - RNA Purification

1. Preheat dry block heaters to 80 °C (Step 2) ± 3 °C as explained in Section-3.
2. Incubate each of the 2 ml microcentrifuge tubes with the RNA-containing supernatant at 80 °C for 30 minutes. Remove the microcentrifuge tubes and cool at ambient 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.

> **NOTE**: RNA Yields and DV_{200} Scores: For downstream NGS applications, a lower concentration of isopropanol may be used to achieve higher DV_{200} scores [3]. Conversely, for maximum RNA yield with the lower DV_{200} 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 ambient temperature. All subsequent centrifugations are performed at ambient 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.

> **NOTE**: **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. This optional on-column DNase digestion must be performed after step 10. See ***Appendix B*** for step-by-step instructions.

11. **2nd wash**:
    a. Add ***650 µl of prepared RNA Wash Buffer*** to the RNA Purification Column.
    b. Centrifuge the assembly at 11,000 x g for 1 minute.

    Discard the flow through and put the Column back into the Collection Tube.
12. **Dry Column**: Centrifuge the Column/Collection Tube assembly at 16,000 x g for 1 minute.

13. **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 ambient temperature.

    c. Centrifuge the Column/RNA Elution Tube assembly at 16,000 x g for 1 minute.

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

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

14. Store the eluted RNA on ice until further processing. Isolated RNA should be kept at −80 °C for long-term storage.

7 - DNA Purification

1. Preheat dry block heaters to 56 °C (Step 8) and 80 °C (Steps 2 and 10) ± 3 °C. Place the heat block adapters in the heat block set to 56°C.
2. Aliquot ***110 µl*** of Buffer BE per sample to be processed into a 1.5 ml microcentrifuge tube. Preheat to 80 °C. If processing more than one sample, multiply the aliquoted volume (110 µl) by the number of samples. Continue to keep the buffer at 80 °C until needed for elution of DNA from column.
3. Prepare ***Tissue Lysis Buffer/PK Solution Mix for DNA*** in a tube following instructions in Table 2 and mix by inverting 10 times or vortexing for 3 seconds.

> **CAUTION**: The Tissue Lysis Buffer/PK Solution Mix should be stored at ambient temperature and used within 30 min after preparation.

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

Table 2. Tissue Lysis Buffer/PK Solution Mix for DNA. *Calculation includes 10% excess in final volume.

4. Open the AFA-TUBE PP Screw-Cap 0.5 ml with the DNA-containing tissue pellet and add ***400 µl*** of the ***Tissue Lysis Buffer/PK Solution Mix*** for DNA. Re-cap the tube tightly.
5. Close the AFA-TUBE PP Screw-Cap 0.5 ml tightly with the Screw-Caps and transfer to the appropriate rack or holder/insert for your Focused-ultrasonicator. Load the rack or holder/insert containing the AFA-TUBE PP Screw-Cap 0.5 ml into the Focused-ultrasonicator for processing.
6. Process sample using the “Acoustic Pellet Resuspension” program.
7. Transfer the AFA-TUBE PP Screw-Cap 0.5 ml from the Focused-ultrasonicator to the AFA-TUBE PP Screw-Cap 0.5 ml Centrifuge and Heat Block adapters on the heat block set to 56 °C.
8. Incubate for a minimum of 60 minutes at 56 °C.

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

9. Remove the AFA-TUBE PP Screw-Cap 0.5 ml with the adapters from the heat block and transfer directly to dry heat block set for 80 °C incubation.
10. Incubate for 60 minutes at 80 °C.
11. Remove the AFA-TUBE PP Screw-Cap 0.5 ml with the adapters from the heat block and let cool for 3 minutes at ambient temperature.
12. Transfer the sample to a 2 ml microcentrifuge tube.

> **NOTE**: **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 ambient temperature, then continue to step 13.

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

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

> **NOTE**: Steps 13 and 14 must be done sequentially, with thorough mixing by vortexing after each addition.

15. Place a DNA Purification Column into a Collection Tube.
16. Transfer ***600 µl*** of sample to the DNA Purification Column.
17. Centrifuge the Column/Collection Tube assembly at 11,000 x g for 1 minute at ambient temperature. All subsequent centrifugations are performed at ambient temperature.
18. Discard the flow-through and place the Column back into the Collection Tube.
19. Repeat steps 16 through 18 until all the sample has passed through the Column.
20. **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.
21. **2nd wash**:
    a. Add ***600 µl of prepared 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.
22. **Dry Column**: Centrifuge the Column/Collection Tube assembly at 16,000 x g for 1 minute.
23. **Elute DNA - 1st step**:
    a. Place the Purification Column into a clean 1.5 ml microcentrifuge tube.
    b. Add ***50 µl*** of pre-warmed Buffer BE (80 °C, from Step 2) to the center of the Column.
    c. Incubate at ambient temperature for 3 minutes.
    d. Centrifuge the Column/microcentrifuge tube assembly at 11,000 x g for 1 minute.

> **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 Buffer BE across the matrix surface.

24. **Elute DNA – 2nd step**:
    a. Add a second 50 µl aliquot of pre-warmed Buffer BE (80 °C) to the center of the Column.
    b. Incubate at ambient 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.

> **NOTE**: For high DNA yield and concentration, the DNA eluate from Step 23d may be reapplied to the column and steps 23b through 23d repeated, omitting Step 24.

25. Isolated DNA should be kept at 2 °C to 8 °C for short term storage (1 to 2 days) and −20 °C for long term storage.

Appendix A: Troubleshooting Guide

Issue	Cause	Solution	Comments / Suggestions
Low yield of 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 (tNA) Ultra 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.	N/A
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.	N/A
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.	N/A
	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.	N/A
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.	N/A
	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.	N/A
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 (tNA) Ultra 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 DNase (2 U/µL) (Thermo Fisher Scientific, PN AM2238), containing 10X TURBO DNase Buffer.

1. Prepare a 65% isopropanol master mix for N samples with 10% excess volume:
   - RNase-free H2O..... 105 µl x N x 1.1
   - 100% Isopropanol ..... 195 µl x N x 1.1
2. Place the RNA Columns into new collection or 2.0 ml microcentrifuge tubes after the 1st wash Step 10 in Section-6 (RNA Purification).
3. Prepare a 1 X TURBO DNase master mix as follows:

Reagent	Volume for N Samples*
RNase-free H2O	93.5 µL x N
10X TURBO DNase buffer	11 µL x N
TURBO DNase	5.5 µL x N

Table 3. DNase Master Mix. *Volumes are 10% in excess.

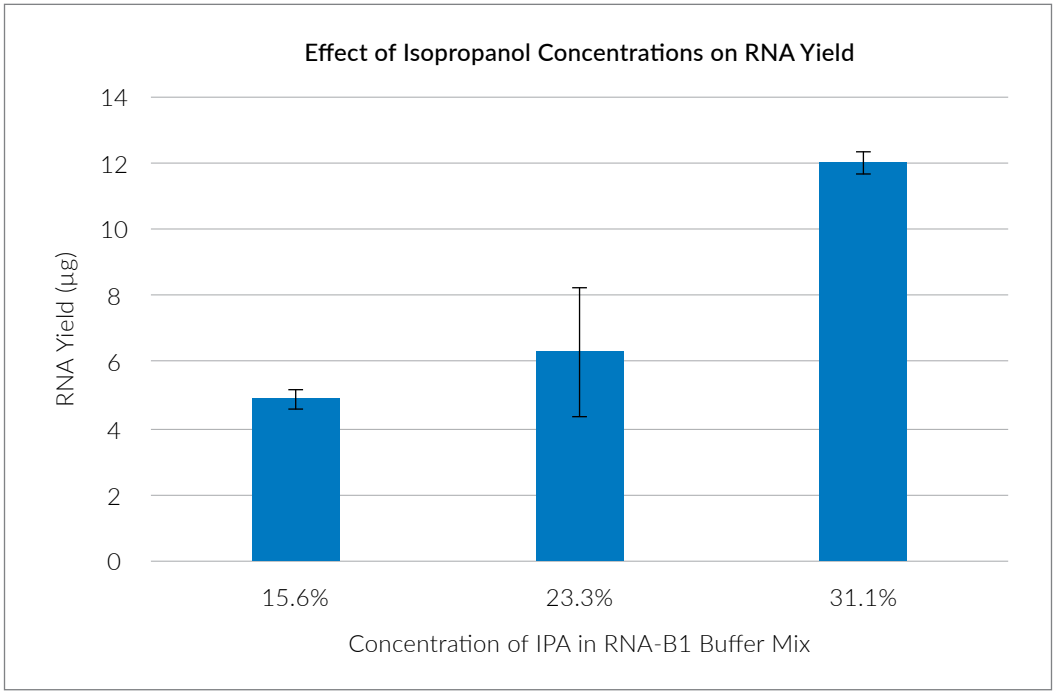
4. Close the cap and gently mix by inverting the tube
5. Add ***100 µl of the DNase master mix*** to the Column and incubate at ambient temperature for 30 minutes.
6. Sequentially add ***175 µl Total NA Plus B1 Buffer*** and then ***300 µl 65% isopropanol*** to the Column.
7. Close cap and vortex to mix.
8. Centrifuge at 11,000 x g for 30 seconds.
9. Pipette the flow-through in the collection tube back into the Column.

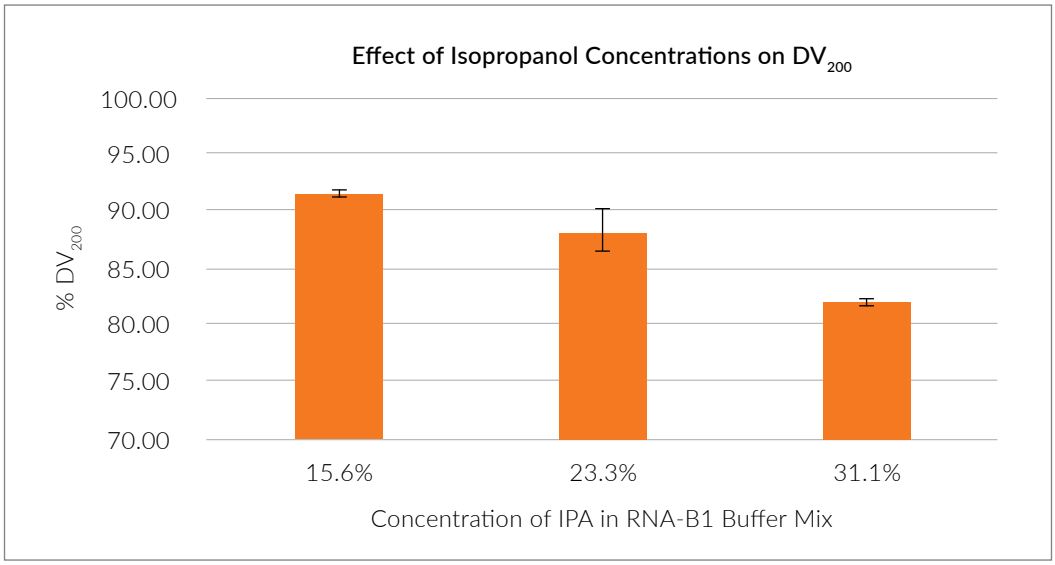
> **CAUTION**: Do not discard the flow-through as it contains the RNA.

10. Centrifuge at 11,000 x g for 30 seconds.
11. Discard the flow-through and place the Column back into the Collection Tube.
12. Proceed with Step 11 in Section-6 (RNA Purification).

Appendix C: Isopropanol Concentration and DV_{200} Scores

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





Final IPA (%)	Stock IPA (%)	IPA Volume (µl)	B1 Buffer Volume (µl)	RNA Volume (µl)
15.6	52.5	350	375	450
23.3	78.3	350	375	450
31.1	100	392	420	450

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:

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

Tips for Determining Quality and Quantity of the Purified FFPE DNA/RNA

- To determine DNA and RNA yields, a fluorometric assay such as Qubit TM (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 or RT-qPCR can be used to assess the amplifiability of isolated DNA and RNA 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 [4].
- Fragment size analysis of the RNA can be used to estimate the quality of the RNA by comparing the %DV200 [5,6]
- RT-PCR can be used to assess the amplifiability of the isolated RNA and correlated to the success of sequencing. [6]

Additional Notes

1. See following link: www.covaris.com/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. Kresse et al. (2018). Evaluation of commercial DNA and RNA extraction methods for high-throughput sequencing of FFPE samples. PLoS ONE 13(5): e0197456.
3. Landolt et al. (2016) RNA extraction for RNA sequencing of archival renal tissues. Scand J Clin Lab Invest 76(5):426-434.
4. 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.
5. Matsubara, T. et al. (2020). DV200 Index for Assessing RNA Integrity in Next-Generation Sequencing. BioMed research interna tional, 2020, 9349132. https://doi.org/10.1155/2020/9349132
6. truXTRAC FFPE RNA Extraction and Purification – Quality Metrics for Clinical Applications Powered by Adaptive Focused Acoustics (AFA) Covaris, 2019