truXTRAC® FFPE total NA (tNA) Plus Kit - Magnetic Beads

(PN 520255)

Part Number: 010451 Rev H | March 2023

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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 at paraffin removal and emulsification from the tissue. Uniquely, AFA enables increased yields of nucleic acids while minimizing 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 30 µm of total FFPE tissue sections or two FFPE cores (1.2 mm in diameter and 5 mm in length). For samples of smaller input sizes, the truXTRAC total NA Kit (PN 520246) may be used for extraction and purification of DNA and RNA from FFPE samples.

This protocol includes optional instructions for storage of FFPE samples in mineral oil. See ***Appendix A*** for more information.

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 cores, 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 C***) in this protocol, check the FAQs found on our website, or contact Covaris Application Support at ApplicationSupport@covaris.com.

Important Notices

1. **Proper Incubation during Proteinase K and De-crosslinking:** Incubation times and temperatures during Proteinase K (at 56 °C) and de-crosslinking (at 80 °C) are crucial to the successful extraction and purification of nucleic acids from FFPE. The recommended process of checking and adjusting temperatures on the heat blocks to ensure proper temperatures of lysate in the vessels must be followed. (see ***Section 3***).

2. **Separation of RNA and DNA containing Fractions by Centrifugation:** A critical step in the truXTRAC FFPE protocol is the separation of RNA and DNA by centrifugation. At this point in the protocol RNA is located in the supernatant and the majority of DNA is still trapped in the tissue pellet. The user must provide equipment that allows centrifugation to protocol specifications (see ***Page 5***). Incomplete separation of fractions can result in yield and quality loss.

3. **FFPE Sample to Sample Variability:** FFPE tissue samples vary widely due to a variety of reasons. The degree of formaldehyde-induced crosslinking, the tissue type itself (highly connective or granular), as well as the wax to tissue ratio can all impact the yield and quality of the extracted and purified nucleic acids. The truXTRAC FFPE protocol was developed to reduce the impact of these factors. However, the user must follow the recommendations (see ***Section 1***) for sample input. In some extreme cases, complete homogenization of the tissue cannot be achieved. After resuspension of the DNA containing pellet and incubation with Proteinase K, chunks of tissue may still be visible. Covaris recommends increasing the Proteinase K incubation time to 90 minutes if the tissue is not completely lysed. The decrosslinking time of RNA and DNA can be increased to 60 and 120 min, respectively.

Kit Contents

- Tissue Lysis Buffer ..... 25 mL
- Proteinase K (PK Solution) ..... 3.5 mL
- Magnetic Bead Suspension ..... 0.5 mL
- Buffer BB3 ..... 75 mL
- Buffer WB3 ..... 60 mL
- Buffer WB4 ..... 60 mL
- RNA Elution Buffer ..... 3.5 mL
- Buffer BE ..... 7.5 mL
- microTUBE-500 AFA Fiber Screw-Cap FFPE ..... 25 count

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

Storage

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

Laboratory Equipment, Chemicals, and Consumables Supplied by User

Required Laboratory Equipment, and Accessories

- microTUBE-500 Centrifuge and Heat Block Adapter (Covaris, PN 500503)
- Dry block heater for 2 mL tubes or temperature-controlled water bath able to accurately heat between 50 to 90 °C
- Magnetic Stand for 2 mL tubes (e.g., Thermo Fisher Scientific, DynaMag-2 Magnet, PN 12321D)

Required Chemicals and Enzymes

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

Optional Enzymes

- TURBO™ DNase (Thermo Fisher Scientific, PN AM2238)
- RNase A, DNase and protease-free (10 mg/mL) (e.g., Thermo Fisher Scientific, PN EN0531)

Required Consumable

- 2 mL nuclease-free microcentrifuge tubes (e.g., Eppendorf Safe-Lock Tubes, PN 022363352)

Covaris Focused-ultrasonicator Accessories and Plate Definitions

The tables 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	LE220RSc
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)	Rack, 24 microTUBE-500 Screw-Cap (500452)
Required Accessories (PN)	Insert XTU (500471)	ME220 Waveguide 4 Place (500534)	N/A	Intensifier (500141)	Intensifier (500141)	N/A	N/A

See ***Appendix D*** for more information.

Instrument	**Plate Definition File Name**
ME220	"4 microTUBE-500 Screw-Cap PN 520185.2.rck"
E220evolution	"500484 E220e 4 microTUBE-500 Screw-Cap -9.9mm offset"
E220 / LE220 / LE220-plus / LE220R-plus / LE220RSc	"Name of Instrument*_500452 Rack 24 Place microTUBE-500 Screw-Cap +6mm offset"

*For example: "LE220plus_500452 Rack 24 Place microTUBE-500 Screw-Cap +6mm offset"

FFPE tNA Extraction and Purification Workflow

Prior to loading, FFPE samples can be stored in mineral oil. See ***Appendix A*** for detailed information.

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 de-crosslinked at 80 °C, and then purified using magnetic beads.

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 using magnetic beads.

1 - FFPE Sample Input Requirements and Guidelines

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

> **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 microTUBE-500
Scalpel or razor blade to scrape material from slides	30 µm of total thickness (e.g., 6 slides at 5 µm thick = 30 µm total thickness) Max Area (on each slide): 100 mm^2

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.

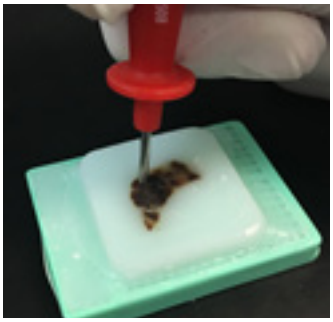
FFPE Curl/Scroll Thickness	Maximum Scrolls per microTUBE-500
5 µm	6
10 µm	3
15 µm	2

Core Input Requirements:

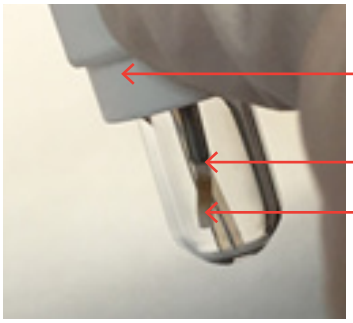
FFPE Core Punch Outer Diameter	Maximum Core Punches per microTUBE-500
≤ 1.2mm (15 Gauge, outer); Length = 5 mm	2

Core punches may be loaded directly into the microTUBE-500 as shown below or transferred into the microTUBE-500 using tweezers or forceps.

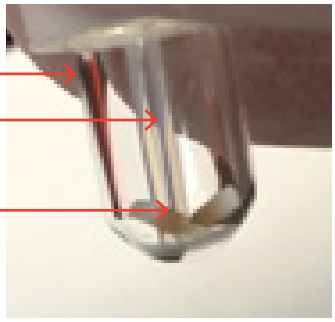
Core Punch from FFPE Block



Loading Core Punch into microTUBE-500



Core Punches Loaded into microTUBE-500



2 - Preparation of Reagents

NOTE: Follow these instructions before starting the FFPE total NA isolation protocol.

1. **Tissue Lysis Buffer:** Visually check for a white precipitate that may form during storage before each use. If white precipitate is visible, incubate the Tissue Lysis Buffer at 50 to 60 °C for 5 to 10 minutes before use to dissolve any precipitate.
2. **80% Ethanol:** Prepare the 80% ethanol solution by mixing 4 parts 100% ethanol with 1 part nuclease-free water. One sample requires 2.3 mL of 80% ethanol. To prepare the total amount of 80% ethanol needed, multiply the number of samples to be processed by 2.6 mL to account for dead volume.

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 a heat block adaptor and a microTUBE-500 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 microTUBE-500 has reached 56 °C or 80 °C ± 3 °C.

CAUTION: The Covaris microTUBE-500 must be used in conjunction with Covaris microTUBE-500 Centrifuge and Heat Block Adapters (PN 500503). It is important to use an accurate heating source for incubation of microTUBE-500 and microcentrifuge tubes during Proteinase K and de-crosslinking incubations. Deviation from 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 5*** 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	E220evolution	E220	LE220 / LE220-plus	LE220RSc
Peak Incident Power (PIP) (Watt)	75	75	200	200	200	450	450
Duty Factor (%)	25	20	10	10	10	20	20
Cycles Per Burst (#)	200	1000	200	200	200	200	200
Treatment time (seconds)	360	360	300	300	300	300	300
Temperature (°C)	20	20	20	20	20	20	20
Water Level (run)	Full	Auto	8	6	6	6	6

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 / LE220-plus	LE220RSc
Peak Incident Power (PIP) (Watt)	75	75	200	200	200	450	450
Duty Factor (%)	25	20	30	30	30	20	30
Cycles Per Burst (#)	200	1000	200	200	200	200	200
Treatment time (seconds)	10	10	10	10	10	10	10
Temperature (°C)	20	20	20	20	20	20	20
Water Level (run)	Full	Auto	8	6	6	6	6

5 - Paraffin Emulsification, Tissue Rehydration, and Lysis

If samples were stored in mineral oil, follow ***Appendix A*** on proper removal prior to continuing.

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

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

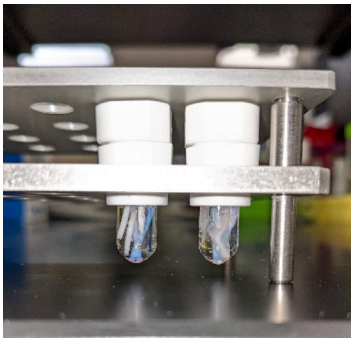
Reagent	Volume for one sample*	Volume for N samples*
Tissue Lysis Buffer	440 µL	440 µL x N
Proteinase K Solution	44 µL	44 µL x N

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

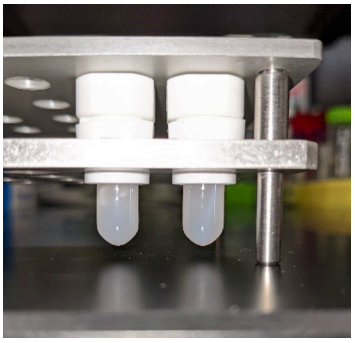
2. Load each FFPE sample into a microTUBE-500.
3. Add 440 µL of the Tissue Lysis Buffer/PK Solution Mix into each microTUBE-500. Ensure that the FFPE sample is fully immersed in the tube to prevent the sample from getting stuck in the Screw-Cap thread.
4. Close the microTUBE-500s tightly with their Screw-Caps and transfer the microTUBE-500s to the appropriate rack or holder/insert for your Focused-ultrasonicator.
5. Load the rack or holder/insert containing the microTUBE-500s 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. See example below.

Before AFA



After AFA



5 - Paraffin Emulsification, Tissue Rehydration, and Lysis (cont.)

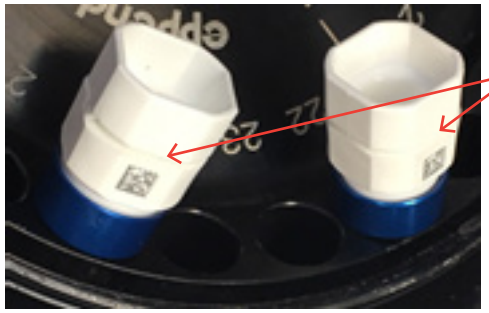
7. Transfer the microTUBE-500s from the Focused-ultrasonicator to the microTUBE-500 Centrifuge and Heat Block adapters.

> **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 samples for 30 minutes at 56 °C. Remove the microTUBE-500s together with the microTUBE-500 adapters from the heat block, and let them cool separately at ambient temperature for 3 min.

> **NOTE:** Do not chill on ice as rapid cooling will cause detergents to precipitate.

9. Place the microTUBE-500s back in the microTUBE-500 adapters. Then, with the bar code on the Screw-Cap sleeve facing outward, transfer microTUBE-500s into a microcentrifuge (fixed angle rotor) and centrifuge at 5,000 x g for 15 minutes.

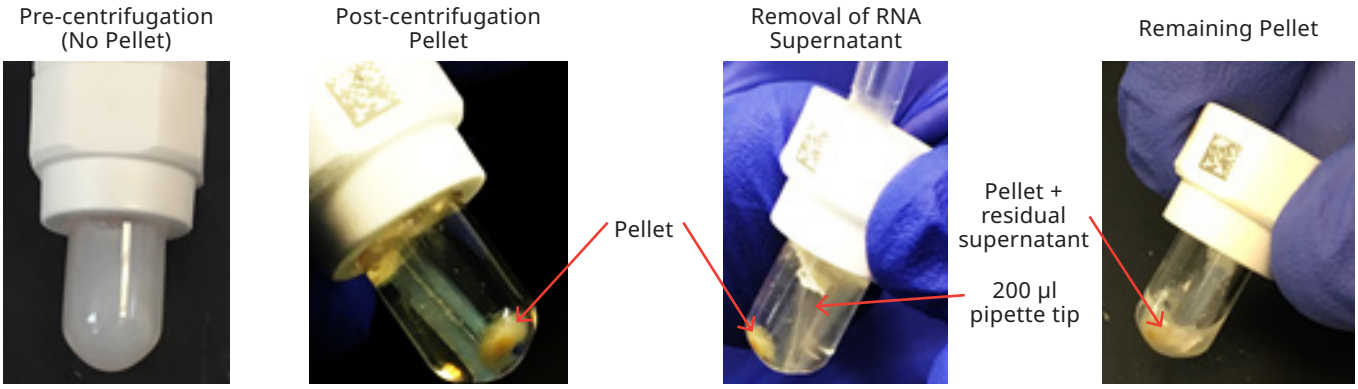


> **NOTE:** The centrifuge lid may not close and may need to be left off during centrifugation

10. Remove microTUBE-500s from microcentrifuge, remove Screw-Cap, and carefully transfer 400 µl of the supernatant into a new 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. Slightly tilt the tube away from the pellet.
> c. Using a 200 µL pipette, 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 towards the tube wall that faces away from the pellet and barcode. Use the same 200 µL pipette a second time to remove the remaining supernatant. ***DO NOT USE WIDE-MOUTH PIPETTE TIPS.***
> d. A layer of emulsified wax may descend obscuring the pellet. This is normal. Leave 20 to 30 µL of supernatant behind. This will not significantly impact RNA or DNA yield.

5 - Paraffin Emulsification, Tissue Rehydration, and Lysis (cont.)



NOTE: If the pellet becomes dislodged from the wall of the microTUBE-500 before the RNA supernatant has been removed,repeat centrifugation ***(Step 8)*** to re-form pellet. Remove RNA supernatant as described in ***Step 9.***

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

NOTE: 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 56 °C (***Steps 6 and 28***) and 80 °C (***Step 2***) 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 tubes and cool at ambient temperature for 3 minutes.
3. Prepare BB3/Magnetic Bead Mix for RNA according to ***Table 2*** below.

Reagent	Volume for one sample*	Volume for N samples*
Buffer BB3	1320 µL	1320 µL x N
Magnetic Bead Suspension	8.8 µL	8.8 µL x N

Table 2. BB3/Magnetic Bead Mix for RNA. *Calculation includes 10% excess in final volume.

> **NOTE:** Vortex the Magnetic Bead Suspension and BB3/Magnetic Bead Mix for RNA before using for 10 seconds to ensure a homogeneous suspension. Beads will settle when left standing.

4. Add 1208 µL of the BB3/Magnetic Bead Suspension mix for RNA to the RNA containing supernatant and cap the microcentrifuge tubes.
5. Vortex the microcentrifuge tubes for 10 seconds.
6. Incubate the microcentrifuge tubes at 56 °C for 10 minutes.
7. Place the tubes on a magnetic stand and incubate for 5 minutes, or until the beads have been pulled to the magnet.

> **NOTE:** With some samples, the binding supernatant may appear slightly brown after the 5 minute incubation on the magnet stand due to a small percentage of beads that do not migrate to the magnet. This effect does not reduce the yield significantly.

8. With the microcentrifuge tubes on the magnetic stand, carefully remove and discard the supernatant. Avoid disturbing the bead pellet.
9. Remove the microcentrifuge tubes from the magnetic stand and add 1 ml of the Buffer WB4.
10. Cap the microcentrifuge tubes and vortex for 10 seconds to resuspend the beads.

> **NOTE:** With some samples, beads may stick to the tube wall and not completely resuspend during vortexing. Proceed to the next step of the protocol even if the beads are not completely resuspended.

11. Place the microcentrifuge tubes back on the magnetic stand and incubate for 5 minutes, or until the beads have been pulled to the magnet.
12. With the microcentrifuge tubes on the magnetic stand, carefully remove and discard the supernatant.

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 DNase digestion must be performed after ***Step 12.*** See ***Appendix B*** for step-by-step instructions.

6 - RNA Purification (cont.)

13. Repeat ***Steps 9 through 12*** for the second wash.
14. After the second wash, remove as much of the supernatant as possible using a 200 µL pipette. Use a 20 µL pipette to remove any remaining liquid from the bottom of the microcentrifuge tubes.

> **NOTE:** It is critical to remove the wash buffer supernatant completely because it contains residual paraffin. Remaining paraffin residue can result in bead clumping during elution and diminished yield.

15. Remove the microcentrifuge tubes from the magnetic stand and add 1 mL of the 80% ethanol solution.
16. Cap the microcentrifuge tubes and vortex for 10 seconds to resuspend the beads.

> **NOTE:** With some samples, beads may stick to the tube wall and not completely resuspend during vortexing. Proceed to the next step of the protocol even if the beads are not completely resuspended.

17. Place the microcentrifuge tubes on the magnetic stand and incubate for 2 minutes, or until the beads have been pulled to the magnet.
18. Remove and discard the supernatant without disturbing the bead pellet.
19. Remove the microcentrifuge tube from the magnetic stand and add 300 µL of the 80% ethanol solution.
20. Cap the microcentrifuge tubes and vortex for 10 seconds.
21. Place the microcentrifuge tubes on the magnetic stand and wait for 2 minutes, or until the beads have been pulled to the magnet.
22. Remove and discard as much of the supernatant as possible. Use a 20 µL pipette to remove any remaining liquid from the bottom of the microcentrifuge tubes.
23. Leave the microcentrifuge tubes uncapped on the magnetic stand for 6 minutes at ambient temperature to let the beads dry.

> **NOTE:** Visually examine that the ethanol has evaporated before continuing with elution. Residual ethanol can inhibit the elution and impact downstream applications such as PCR.

24. Remove the microcentrifuge tubes from the magnetic stand and add 50 to 100 µL of the RNA Elution Buffer. Resuspend the beads by pipetting up and down 20 times. Ensure that all beads are resuspended in the buffer.
25. Cap the microcentrifuge tubes and incubate in the heat block set to 56 °C for 5 minutes.
26. Transfer the microcentrifuge tubes from the heat block to the magnetic stand and incubate for 2 minutes.
27. Transfer the eluate into a new/clean microcentrifuge tube without transferring beads. A small amount of residual paraffin may be visible in the pipet tip. This will not adversely affect downstream processing of the eluted RNA.
28. 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 (***Steps 7, 15, and 34***) and 80 °C (***Step 9***) ± 3 °C. Place the heat block adapters in the heat block set to 56 °C.
2. Prepare the Tissue Lysis Buffer/PK Solution Mix for DNA in a microcentrifuge tube following instructions in ***Table 3***. Mix by inverting 10 times or vortexing for 3 seconds.

> **NOTE:** The Tissue Lysis Buffer/PK Solution Mix should be stored at ambient temperature and used within 30 minutes 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 3. Tissue Lysis Buffer/PK Solution Mix for DNA. *Calculation includes 10% excess in final volume.

3. Add 400 µL of the Tissue Lysis Buffer/PK Solution Mix for DNA into each microTUBE-500 containing the DNA-tissue pellet.
4. Close the microTUBE-500s 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 microTUBE-500s into the instrument for processing.
5. Process sample using the "***Acoustic Pellet Resuspension***" program.
6. Transfer the microTUBE-500s from the Focused-ultrasonicator to the microTUBE-500 Centrifuge and Heat Block adapters on the heat block set to 56 °C.
7. Incubate for a minimum of 60 minutes at 56 °C.

> **NOTE:** Proteinase K-treated samples can be stored at ambient 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 core punch samples.

8. Remove the microTUBE-500s with the adapters from the heat block and transfer directly to dry heat block set for 80 °C incubation.
9. Incubate for 60 minutes at 80 °C.
10. Remove the microTUBE-500s with the adapters from the heat block and cool separately for 3 minutes at ambient temperature.
11. Transfer the samples to a 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 ambient temperature, then continue to ***Step 12***.

7 - DNA Purification (cont.)

12. Prepare the BB3/Magnetic Bead Mix according to ***Table 4***.

Reagent	Volume for one sample*	Volume for N samples*
Buffer BB3	792 µL	792 µL x N
Magnetic Bead Suspension	8.8 µL	8.8 µL x N

Table 4. BB3/Magnetic Bead Mix for DNA. *Calculation includes 10% excess in final volume.

> **NOTE:** Vortex the Magnetic Bead Suspension and BB3/Magnetic Bead Mix before using for 10 seconds to ensure a homogeneous suspension. Beads will settle when left standing.

13. Add 728 µL of the BB3/Magnetic Bead Mix to each of the DNA solutions in 2 mL microcentrifuge tubes.
14. Cap the microcentrifuge tubes and vortex for 10 seconds.
15. Incubate the microcentrifuge tubes at 56 °C for 5 minutes.
16. Place the microcentrifuge tubes on a magnetic stand and wait for 5 minutes or until the beads have been pulled to the magnet.

> **NOTE:** With some samples, the binding supernatant may appear slightly brown after the 5-minute incubation on the magnet stand due to a small percentage of beads that do not migrate to the magnet. This effect does not reduce the yield significantly.

17. With the microcentrifuge tubes still on the magnetic stand, carefully remove and discard the supernatant. Avoid disturbing the bead pellet.
18. Remove microcentrifuge tubes from the magnetic stand and add 1 mL of the Buffer WB3.
19. Cap the microcentrifuge tubes and vortex for 10 seconds to resuspend the beads.

> **NOTE:** With some samples, beads may stick to the tube wall and not completely resuspend during vortexing. Proceed to the next step of the protocol even if the beads are not completely resuspended.

20. Place the microcentrifuge tubes back on the magnetic stand and wait for 5 minutes, or until the beads have been pulled to the magnet.
21. With the microcentrifuge tubes still on the magnetic stand, carefully remove and discard the supernatant.
22. Repeat wash ***Steps 18 through 21***.
23. After the final wash, remove as much of the supernatant as possible. Use a 20 µL pipette to remove any remaining liquid from the bottom of the tube.

> **NOTE:** It is critical to remove the wash buffer supernatant completely because it contains residual paraffin. Remaining paraffin residue will result in bead clumping during elution and diminished yield.

24. Remove the microcentrifuge tubes from the magnetic stand and add 1 mL of the 80% ethanol solution to the tubes.

7 - DNA Purification (cont.)

25. Cap the microcentrifuge tubes and vortex for 10 seconds to resuspend the beads.

> **NOTE:** With some samples, beads may stick to the tube wall and not completely resuspend during vortexing. Proceed to the next step of the protocol even if the beads are not completely resuspended.

26. Place the microcentrifuge tubes on the magnetic stand and wait for 2 minutes, or until the beads have been pulled to the magnet.
27. Remove and discard the supernatant without disturbing the bead pellet.
28. Remove the microcentrifuge tubes from the magnetic stand and add 300 µL of the 80% ethanol solution.
29. Cap the microcentrifuge tubes and vortex for 10 seconds.
30. Place the microcentrifuge tubes on the magnetic stand and wait for 2 minutes, or until the beads have been pulled to the magnet.
31. Remove and discard as much of the supernatant as possible. Use a 20 µL pipet to remove any remaining liquid from the bottom of the tube.
32. Leave the microcentrifuge tubes uncapped on the magnetic stand and let the beads dry for 6 minutes at ambient temperature.

> **NOTE:** Visually examine that the ethanol has evaporated before continuing with elution. Residual ethanol can inhibit the elution and impact downstream applications such as PCR.

33. Remove the microcentrifuge tubes from the magnetic stand and add 50 to 100 µL of Buffer BE (5 mM TrisCl pH 8.5) into the tube.
34. Re-suspend the beads by pipetting up and down 20 times. Ensure that all beads are submerged in the buffer and are fully suspended.
35. Cap the microcentrifuge tubes and incubate them in the heat block set to 56 °C for 5 minutes.
36. Remove the microcentrifuge tubes from the heat block and place it on the magnetic stand and wait for 2 minutes, or until the beads have been pulled to the magnet.
37. Transfer the eluate into a clean/new microcentrifuge tube avoiding transfer of beads. A small amount of residual paraffin may be visible in the pipet tip. This will not adversely affect downstream processing of the eluted DNA.
38. 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: Optional Pre-treatment for Mineral Oil Stabilized FFPE

Introduction

The Pre-Treatment for Mineral Oil-Stabilized FFPE is specifically designed to prepare FFPE tissue samples such as scrolls, slide scrapings and cores that are submerged in mineral oil for downstream extraction of nucleic acids. Once an FFPE sample is cut from the main block, storage in mineral oil can prevent unwanted oxidation of biomolecules during storage and shipment. Thus, the sample can be shipped or stored safely until extraction of biomolecules at the laboratory site. The mineral oil removal workflow also ensures that excessive wax/paraffin is partially removed, thereby reducing the need for tedious trimming of wax from the tissue block.

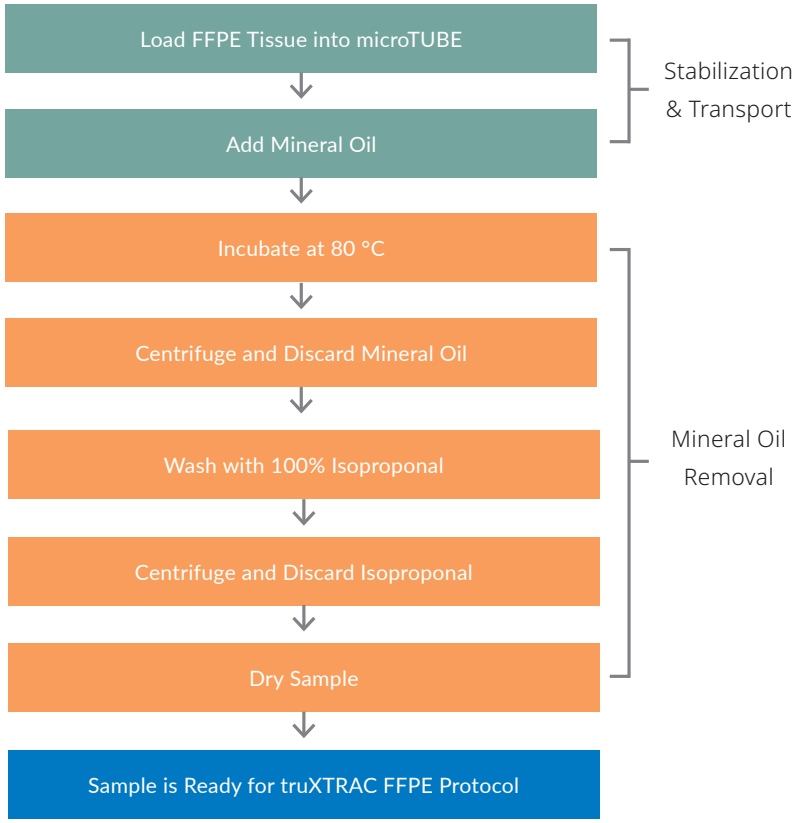
This protocol is fully compatible with downstream extraction of total nucleic acids (tNA) from Formalin-Fixed Paraffin-Embedded (FFPE) tissue samples using Covaris Adaptive Focused Acoustics (AFA).

Note that the FFPE sample input requirements are different than the standard, no mineral oil preserved samples. The maximal amount of tissue per microtube increases with the addition of mineral oil. But for very low tissue amounts, the addition of mineral oil can result in reduced RNA recovery.

Required Additional Reagents

- Mineral oil, light oil for molecular biology (e.g. Sigma-Aldrich, PN 69794)
- 100% Isopropanol, ultrapure (e.g. AmericanBio, PN AB07015)

Procedure Workflow Overview



Appendix A: Optional Pre-treatment for Mineral Oil Stabilized FFPE (cont.)

Mineral Oil Stabilized FFPE Sample Input Requirements and Guidelines

> **CAUTION:** Do NOT go outside of the input requirements in the tables below. Doing so will negatively impact the quality and quantity of extractable nucleic acids.

Inputs listed as per microTUBE-500. A minimum of 2.0 mg of tissue is recommended.

Slide Section Input Requirements:

Slide Collection Method	Minimum Input	Maximum Input
Scalpel or razor blade to scrape material from slides	15 µm of total sample thickness (e.g., 3 slides at 5 µm thick = 15 µm total thickness) Min Area (on each slide): 7.5 mm^2	40 µm of total sample thickness (e.g., 8 slides at 5 µm thick = 40 µm total thickness) Max Area (on each slide): 15 mm^2

Curls/Scrolls Input Requirements:

FFPE Curl/Scroll Thickness	Minimum Input	Maximum Input
5 µm	2	10
10 µm	1	5
15 µm	1	3

Core Input Requirements:

FFPE Core Punch Diameter	Minimum Input	Maximum Input
≤ 1.2mm; Length = 5 mm	1	2

Sample Storage in Mineral Oil:

1. Load each FFPE sample into a microTUBE-500.
2. Add 400 µL of mineral oil into each microTUBE-500. Ensure that the FFPE sample is fully immersed in the tube to prevent the sample from getting stuck in the Screw-Cap thread.
3. Close the microTUBE-500s tightly with their Screw-Caps.

> **NOTE:** Samples can now be stored and shipped until extraction.

Removal of Mineral Oil:

Mineral oil removal should be done just before commencing with the truXTRAC FFPE workflow to prevent oxidation-based aging of the bare tissue samples. Before beginning, follow ***Sections 2 through 4*** in the main protocol.

1. Preheat dry block heaters to 56 °C (Step 13) and 80 °C (Step 3) ± 3 °C as explained in ***Section 3***.
2. Place the microTUBE-500s into the microTUBE-500 Centrifuge and Heat Block adapters.
3. Incubate the microTUBE-500s at 80 °C for 5 minutes.
4. Vortex briefly for 3 seconds.

Appendix A: Optional Pre-treatment for Mineral Oil Stabilized FFPE (cont.)

5. Place the microTUBE-500s in the microTUBE-500 adapters. Then, with the bar code on the Screw-Cap sleeve facing outward, transfer microTUBE-500s into a microcentrifuge (fixed angle rotor) and centrifuge at 5,000 x g for 10 minutes.
6. Remove microTUBE-500s from microcentrifuge, remove Screw-Cap, and carefully remove all of the supernatant with a 200 µL pipette tip.

> **CAUTION:** For guidelines for removing supernatant from the microTUBE-500, read the Caution after ***Section-5 Step 10.*** Depending on the tissue collection and input amount, the tissue could present as a small pellet or large pieces of tissue.

7. Add 400 µL of 100% isopropanol.
8. Vortex briefly for 3 seconds.
9. Place the microTUBE-500s in the microTUBE-500 adapters. Then, with the bar code on the Screw-Cap sleeve facing outward, transfer microTUBE-500s into a microcentrifuge (fixed angle rotor) and centrifuge at 5,000 x g for 10 minutes.
10. Remove microTUBE-500s from microcentrifuge, remove Screw-Cap, and carefully remove all of the supernatant with a 200 µL pipette tip.
11. Use a 20 µL pipette to remove any remaining liquid from the bottom without disturbing the tissue.
12. Leave the microTUBE-500s Screw-Caps off.
13. Place the opened microTUBEs into a 56 °C heat block with the Screw-Caps removed for 10 minutes to evaporate the isopropanol.
14. Proceed with ***Section-5***.

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. This procedure is done after ***Step 12*** in ***Section-6*** (RNA Purification).

The protocol below describes removal of DNA specifically using TURBO DNA-free kit (ThermoFisher Scientific PN AM1907).

1. Use ***Table 5*** to Prepare a 1X TURBO DNase master mix:

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 5. DNase Master Mix. *Calculation includes 10% excess in final volume.

2. Add 100 µL of DNase master mix to each bead pellet.
3. Re-suspend the beads by pipetting up and down 20 times.
4. Incubate at ambient temperature for 30 minutes.
5. Add 300 µL of Buffer BB3 and vortex for 5 seconds.
6. Incubate at ambient temperature for 10 minutes.
7. Place the microcentrifuge tubes on a magnetic stand and incubate for 5 minutes or until the beads have been pulled to the magnet.
8. Carefully remove the supernatant using a 200 µL pipette. Avoid disturbing the bead pellet.
9. Proceed with ***Step 13*** in ***Section-6*** (RNA Purification).

Appendix C: Troubleshooting Guide

Issue	Cause	Solution	Comments / Suggestions
Low yield of DNA	First proteinase K incubation too long	Optimize the 1st proteinase K digestion step for your tissue samples.	During the 1st incubation step with proteinase K at 56 °C, the RNA is released, and most of the DNA stays in the remaining tissue. If the PK digestion step is too long, the tissue will be over digested resulting in the release of the DNA into the solution.
	Parts or entire tissue pellet lost during supernatant removal	Repeat using narrow mouth 200 µL pipette tip to take off RNA-containing supernatant or switch to a 20 µL pipette tip to finish removing the supernatant.	Follow guidelines in the protocol closely. Make sure laboratory personnel are trained in this procedure.
	Loss of magnetic beads during purification steps	Remove supernatant of bind and wash steps slowly and carefully. If beads appear in the pipet tip, eject the liquid back into the tube, wait for 1 minute, and try aspirating the supernatant again.	The viscosity of Buffers BB3, WB3, and WB4, as well as the presence of the paraffin emulsion, can make supernatant removal difficult.
	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 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.***
Low yield of RNA	Loss of magnetic beads during purification steps	Remove supernatant of bind and wash steps slowly and carefully. If beads appear in the pipet tip, eject the liquid back into the tube, wait for 1 minute, and try aspirating the supernatant again.	The viscosity of Buffers BB3, WB3, and WB4, as well as the presence of the paraffin emulsion, can make supernatant removal difficult.
	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 kit use FFPE blocks that have been well characterized for yield and quality.
Eluates are cloudy	Residual paraffin in elution	Spin the eluate for 30 seconds at 10,000 rcf. The residual wax will form a layer on top of the liquid and the aqueous solution can be transferred to a new tube.	If the paraffin emulsion was not completely removed in the wash steps, residual wax can be carried through to the elution step.
FFPE Sample to Sample Variability	Possible causes include degree of formaldehyde-induced crosslinking, the tissue type itself, and ratio of wax to tissue	Follow recommendations of sample input (***Page 7***).	If chunks of tissue are still visible, increase Proteinase K incubation times to 90 minutes and decrosslinking of RNA to 60 and 120 min, respectively.

Appendix D: Removing or Installing the Intensifier (Covaris PN 500141) from a Covaris E System

The 500141 Intensifier is a small, inverted stainless-steel cone centered over the E-Series transducer by four stainless wires. The wires are held in place by a black plastic ring pressed into the transducer well.

If an AFA protocol requires "no Intensifier", please remove the Intensifier, using the following steps:

1. Empty the water bath. Start the instrument and start the SonoLab software.
2. Wait for the homing sequence to complete (the transducer will be lowered with the rack holder at the home position, allowing easy access to the Intensifier).
3. Grasp opposite sides of plastic ring and gently pull the entire assembly out of the transducer well. Do not pull on the steel cone or the wires. The ring is a friction fit in the well – no hardware is used to hold it in place.

The 500141 Intensifier (left) shown installed in the E-Series transducer well and (right) removed. Note the "UP" marking at the center of the Intensifier.

If a protocol requires the Intensifier to be present, simply reverse this process:

4. Align the black plastic ring with the perimeter of the transducer well. Note that the flat side of the center cone (marked UP) should be facing up (away from the transducer).
5. Gently press each section of the ring into the well until the ring is seated uniformly in contact with the transducer, with approximately 2 mm of the ring evenly exposed above the transducer assembly. Do not press on the cone or wires. The rotation of the ring relative to the transducer assembly is not important.
6. Refill the tank. Degas and chill the water before proceeding.

Technical Assistance

Technical Support – Ongoing assistance with the operation or application of the equipment and/or troubleshooting is provided via:

- Telephone
  - United States: Tel: +1 781.932.3959
  - Europe: Tel: 44 (0) 845 872 0100
- E-mail instrumentation queries to techsupport@covaris.com or application queries to applicationsupport@covaris.com

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 of 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) that can occur. 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 the following link: https://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. 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.

Support and Technical Assistance

Tech Support: Ongoing assistance with the operation or application of the equipment and/or troubleshooting is provided via:

- **Telephone**:
  - US & APAC: +1 781.932.3959
  - EU: +44 (0)845 872 0100
- **E-mail:**
  - Service and Instrumentation: techsupport@covaris.com
  - Solutions: applicationsupport@covaris.com
  - US Customer Service: customerservice@covaris.com
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