truXTRAC® FFPE total NA (tNA) Kit - Magnetic Bead Purification (25)

Adaptive Focused Acoustics® (AFA®)-based sequential RNA and DNA Extraction from FFPE Tissues using Magnetic Bead-based Purification

PN 520246

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General Information

Intended Use

The truXTRAC FFPE total NA (Nucleic Acid) 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 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 slide sections up to 20 µm in total material thickness (from a 10 mm^2 area), scrolls/curls up to 20 µm in total material thickness, and one core up to 1.4 mm in diameter. For samples of larger input sizes, the truXTRAC total NA Plus Kit (PN 520255) using magnetic bead-based purification 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 cores, 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.

Note for users:

If you require any assistance with this product, please refer to Troubleshooting (***Appendix B***) 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
010445	A	6/2018	Kit Release of truXTRAC FFPE total NA Kit – Magnetic Beads
010445	B	7/2018	Update protocol with new clarifications
010445	C	8/2019	Removed tissuePICK, sectionPICK and related accessories
010445	D	11/2019	Updated RNA purification and DNase treatment protocols
010445	E	1/2021	Updated typographical errors

Kit Contents

- Tissue Lysis Buffer.. 6 ml
- Proteinase K (PK Solution).. 1.25 ml
- Magnetic Bead Suspension .... 0.5 ml
- Buffer BB3.. 25 ml
- Buffer WB3 .... 38 ml
- Buffer WB4 .... 38 ml
- RNA Elution Buffer .... 3.5 ml
- Buffer BE .... 7.5 ml
- microTUBE-130 AFA Fiber Screw-Cap FFPE .... 25 tubes

SDS Information available at: https://www.covaris.com/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 room temperature.

Laboratory Equipment, Chemicals, and Consumables Supplied by User

Required Laboratory Equipment and Accessories

- microTUBE-130 Centrifuge and Heat Block Adapter (PN 500406)
- Magnet Stand for 2 ml tubes (e.g., Thermo Fisher Scientific, DynaMag™-2 Magnet, PN 12321D)
- Dry block heater with block to accommodate 2 ml tubes or temperature-controlled water bath able to accurately heat between 50 to 90 °C

Required Reagents

- 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)
- Optional DNase-free RNase A (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 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.

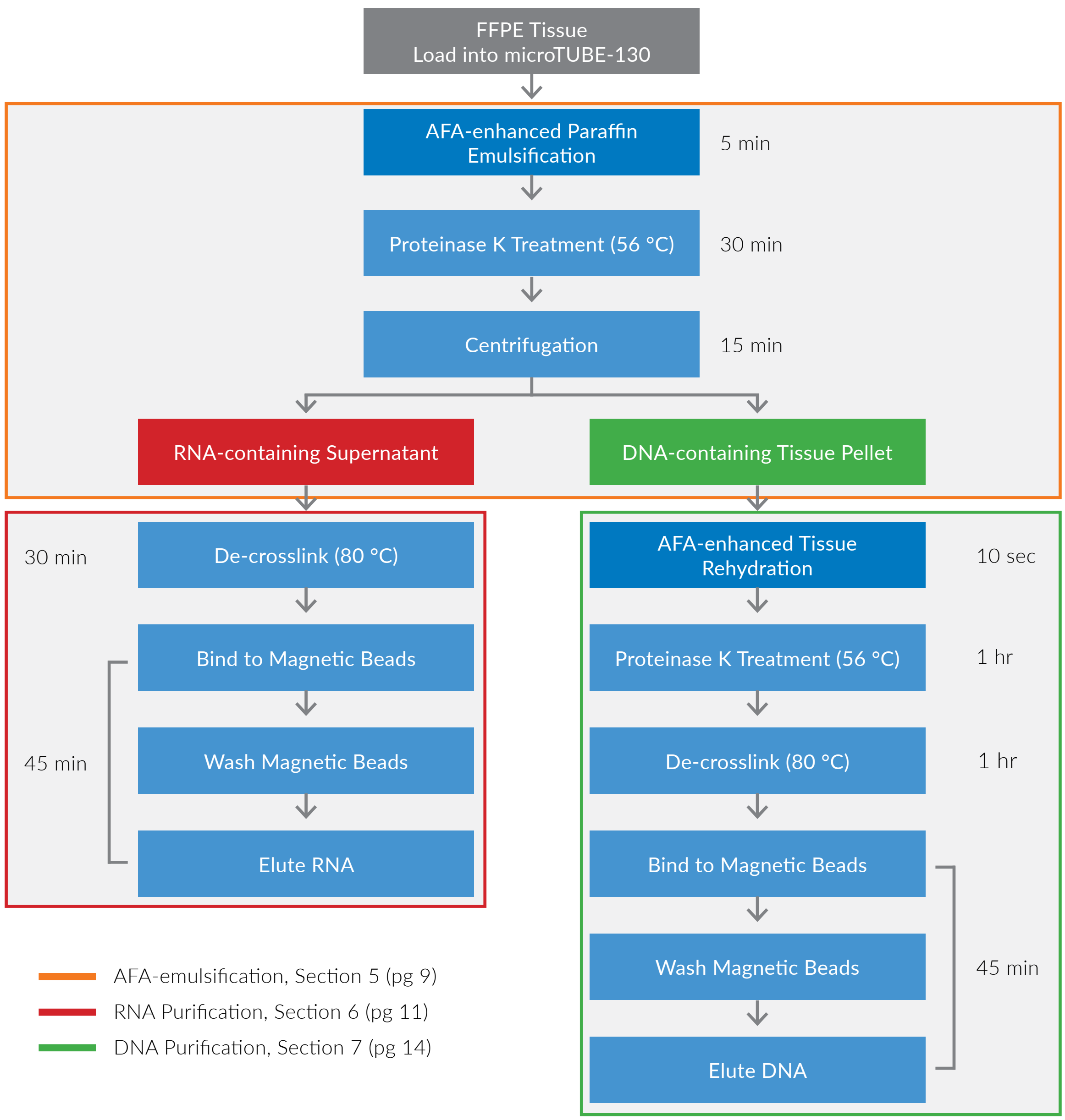
Instrument	M220	ME220	S220	E220*evolution*	E220	LE220/LE220-plus
Holder/Rack Description (PN)	Holder XTU (500414)	Rack 4-place microTUBE Screw-Cap (500522)	Holder microTUBE Screw-Cap (500339)	Rack E220e 4 Place microTUBE Screw Cap (500432)	Rack 24 Place microTUBE Screw-Cap (500308)	Rack 24 Place microTUBE Screw-Cap (500308)
Plate Definition File Name	N/A	<4 microTUBE-130 Screw-Cap PN 520216>	N/A	<500432 Rack E220e 4 Place microTUBE Screw Cap>	<500308 Rack 24 Place microTUBE Screw-Cap>	<500308 Rack 24 Place microTUBE Screw-Cap>
Required Accessories (PN)	Insert XTU (500489)	ME220 Waveguide 4 Place (500534)	N/A	Intensifier (500141)	Intensifier (500141)	N/A

FFPE RNA Extraction and Purification Workflow

Using the AFA process, FFPE tissue 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 using magnetic beads.

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

Scalpel or razor blade collection

Slide Collection Method	Maximum Input per microTUBE
Scalpel or razor blade to scrape material from slides	20 µm of total thickness Area: 10 x 10 mm (2 slides at 5 µm thick = 10 µm total thickness)

Curls/Scrolls Input Requirements:

For best results, minimize the amount of wax present by trimming. We recommend no more than 1-part wax to 1-part tissue.

FFPE Curl/Scroll Thickness	Maximum Scrolls per microTUBE-130
5 µm	3
10 µm	2
15 to 20 µm	1

Core Input Requirements:

FFPE Core Punch Outer Diameter	Maximum Core Punches per microTUBE-130
≤ 1.4mm (15 Gauge, outer); Length = 5 mm	1

2 - Preparation of Reagents

Note: Follow these instructions before starting the FFPE total NA isolation process.

1. **80% Ethanol:** prepare 80% ethanol by mixing 4 parts 100% ethanol with 1 part nuclease free water. One sample requires 1.8 ml of 80% ethanol. To prepare the total amount of 80% ethanol needed, multiply the number of samples to be processed by 2 ml.

3 - Preparation of Heat Blocks

1. Preheat dry block heaters to 56 °C and 80 °C ± 3 °C. It is critical that these temperatures are accurate in order to successfully execute the protocol.
2. Test the temperature of your 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 56 °C or 80 °C ± 3 °C.

> **CAUTION:** The Covaris microTUBE must be used in conjunction with Covaris Centrifuge and Heat Block microTUBE Adapters (PN 500406). It is important to use an accurate heating source for incubation of microTUBE-130s 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.

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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 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 / LE220-plus
Peak Incident Power (PIP) (Watt)	75	75	175	175	175	450
Duty Factor (%)	20	25	10	10	10	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	18	18	18	18
Water Level (run)	Full	Auto	15	10	10	15

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
Peak Incident Power (PIP) (Watt)	75	75	175	175	175	450
Duty Factor (%)	20	25	10	10	10	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	18	18	18	18
Water Level (run)	Full	Auto	15	10	10	15

5 - Paraffin Emulsification, Tissue Rehydration, and Lysis

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

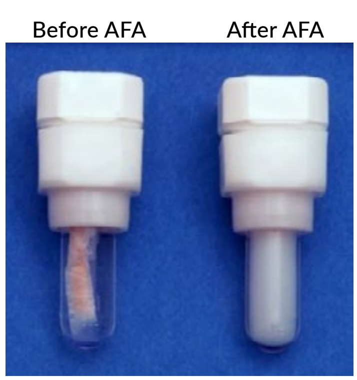
> **CAUTION:** The Tissue Lysis Buffer/Proteinase K Mix should be stored at room temperature and used within 30 min after preparation.

Reagent	Volume for one sample*	Volume for N samples*
Tissue Lysis Buffer	121 µl	121 µl x N
Proteinase K Solution	11 µl	11 µl x N

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

2. Open the microTUBE-130 Screw-Cap and load the FFPE tissue.
3. Add 120 µl Tissue Lysis Buffer/Proteinase K mix to the microTUBE-130. FFPE tissue may also be added directly to microTUBEs containing Lysis buffer. If adding FFPE tissue to microTUBEs containing Lysis buffer, 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-130 tightly with the Screw-Cap and transfer the microTUBE-130 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.



6. Remove the microTUBE-130 from the Focused-ultrasonicator and load it into the microTUBE-130 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 56 °C (Step 7).

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

7. Incubate for 30 minutes at 56 °C. Remove microTUBE-130 together with the microTUBE-130 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-130 in the microTUBE-130 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.

9. Open the microTUBE and carefully transfer 100 µl supernatant into a clean 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 10 to 20 µl of supernatant behind. This will not significantly impact RNA or DNA yield.

10. Save the DNA-containing tissue pellet in the microTUBE 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 to 8 °C for up to 1 day. For longer periods, store at –15 to –30 °C.

6 - RNA Purification

1. Set up the dry-heat blocks as explained in Section-3 and verify that the block temperatures have reached 80 °C (Step 2) and 56 °C (Step 22).
2. Incubate 2 ml microcentrifuge tube with the RNA-containing supernatant at 80 °C for 20 minutes. Remove tubes from the heat block and cool at room temperature for 3 minutes.
3. Prepare BB3/Magnetic Bead Mix according to Table 2 below.

Reagent	Volume for one sample*	Volume for N samples*
BB3	330 µl	330 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.

CAUTION: Thoroughly vortex the Magnetic Bead Suspension and BB3/Magnetic Bead Mix before using to ensure a homogenous suspension. Beads will settle when left standing.

4. Add 308 µl of the BB3/Magnetic Bead Mix to the RNA-containing supernatant and cap the microcentrifuge tube.
5. Vortex the microcentrifuge tube for 10 seconds.
6. Incubate the microcentrifuge tube at ambient temperature for 10 minutes
7. Place the tube on a magnetic stand and incubate for 5 minutes until the beads have been pulled to the magnet.

CAUTION: With some samples, the binding supernatant will 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 tube on the magnet, carefully remove and discard the supernatant using a 200 µl pipette. Avoid touching or disturbing the bead pellet.
9. Remove the microcentrifuge tube from the magnetic stand and add 600 µl WB4.
10. Cap the tube and vortex for 10 seconds. Confirm that all beads are resuspended. If beads are still sticking to the wall continue vortexing until all beads are resuspended.
11. Place the tube back on the magnetic stand and incubate for 5 minutes until the beads have been pulled to the magnet.

6 - RNA Purification (cont.)

12. With the tube on the magnet, carefully remove and discard the supernatant. Remove as much of the supernatant as possible using a 200 µl pipette. Use a 20 µl pipette to remove the remaining liquid from the bottom of the tube.

> **CAUTION:** 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.

Optional step recommended for FFPE core sample inputs: Repeat wash steps 8 through 11. The beads may not fully re-suspend in the 2nd wash with WB4 which does not present a problem. If you perform the optional DNase below, this includes a 2nd wash step and is not necessary here.

Optional DNA removal step: The truXTRAC FFPE total NA 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 DNase digestion must be performed after Step 11. See ***Appendix B*** for step-by-step instructions.

13. Remove the tube from the magnetic stand and add 600 µl 80% ethanol.
14. Cap the tube and vortex for 10 seconds. Confirm that all beads are resuspended. If beads are still sticking to the wall continue vortexing until all beads are resuspended.
15. Place the tube on the magnetic stand and incubate for 2 minutes until the beads have been pulled to the magnet.
16. Remove and discard the supernatant without disturbing the bead pellet.
17. Remove the tube from the magnetic stand and add 300 µl 80% ethanol.
18. Cap the tube and vortex for 10 seconds. Confirm that all beads are resuspended. If beads are still sticking to the wall continue vortexing until all beads are resuspended.
19. Place the tube on the magnetic stand and incubate for 2 minutes until the beads have been pulled to the magnet.
20. Remove and discard as much of the supernatant as possible. Use a 20 µl pipette to remove the remaining liquid from the bottom of the tube.

> **CAUTION:** Make sure that the ethanol has evaporated before continuing with elution. Residual ethanol can inhibit the elution and impact downstream applications such as PCR.

20. Leave the tube open on the magnetic stand and let the beads dry for 6 minutes at room temperature.
21. Remove the tube from the magnetic stand and add 50 to 100 µl of RNA Elution Buffer. Resuspend the beads by pipetting up and down 20 times. Ensure that all of the beads are resuspended in the buffer and none are still sticking to the wall of tube.
22. Cap the tube and incubate it in the heat block set to 56 °C for 5 minutes.
23. Remove the tube from the heat block and place it on the magnetic stand and incubate for 2 minutes until the beads have been pulled to the magnet.
24. Transfer the eluate into a clean elution tube without transferring beads. A small amount of residual paraffin may be visible in the pipette tip. This will not adversely affect downstream processing of the eluted RNA. Keep eluted RNA on ice until further processing. Isolated RNA should be kept at -80 °C for long term storage.

7 - DNA Purification

1. Set up the dry-heat blocks as explained in Section-3 and verify the block temperatures to be 56 °C and 80 °C. The heat block set to 56 °C is required for Proteinase K incubation (Step 7) and DNA elution after purification via magnetic beads (Step 33). The heat block at 80 °C is required for DNA de-crosslinking (Step 9). Place the heat block adapters in the heat block set to 56 °C.
2. Prepare Tissue Lysis Buffer/Proteinase K Mix in a tube following the instructions in Table 3 and mix by inverting 10 times or vortexing for 3 seconds.

> **CAUTION:** Make sure that the ethanol has evaporated before continuing with elution. Residual ethanol can inhibit the elution and impact downstream applications such as PCR.

Reagent	Volume for one sample*	Volume for N samples*
Tissue Lysis Buffer	88 µl	88 µl x N
Proteinase K	22 µl	22 µl x N

Table 3. Tissue Lysis Buffer/Proteinase K Mix for DNA
*Calculation includes 10% excess in final volume.

3. Open the microTUBE with the DNA-containing tissue pellet (Section-5 step 10) and add 100 µl of the Tissue Lysis Buffer/Proteinase K Mix for DNA. Re-cap the Screw-Cap microTUBE tightly.
4. Transfer the microTUBE-130 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 Pellet Resuspension***" program on your Covaris Focused-ultrasonicator.
6. Remove the microTUBE-130 from the Focused-ultrasonicator and load the microTUBE into the pre-warmed microTUBE-130 Centrifuge and Heat Block adapter on the heat block set to 56 °C.
7. Incubate for 60 minutes at 56 °C.

   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 Proteinase K incubation time to 2 hours or up to overnight for >15 µm scrolls or 1.4 mm core sample inputs.

8. Remove microTUBE together with the microTUBE-130 adapter from the heat block and transfer directly to the dry heat block set-up for 80 °C incubation.
9. Incubate for 60 minutes at 80 °C.
10. Remove the microTUBE-130 from the heat block and let cool for 3 minutes at room temperature. Do not chill on ice.
11. Transfer the entire sample into 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 solution and incubate for 5 minutes at room temperature, then continue to step 12.

7 - DNA Purification (cont.)

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

Reagent	Volume for one sample*	Volume for N samples*
BB3	198 µl	198 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.

> **CAUTION:** Thoroughly vortex the Magnetic Bead Suspension and BB3/Magnetic Bead Mix before using to ensure a homogenous suspension. Beads will settle when left standing.

13. Add 188 µl of the BB3/Magnetic Bead Mix to the DNA solution in the 2 ml tube.
14. Cap the tube and vortex the tube for 10 seconds.
15. Incubate the tube on the benchtop at room temperature for 10 minutes to ensure complete binding of the DNA.
16. Place the tube on the magnetic stand and incubate for 5 minutes until the beads have been pulled to the magnet.

> **CAUTION:** 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 tube still on the magnet, carefully remove and discard the supernatant using a 200 µl pipette. Avoid touching or disturbing the bead pellet.
18. Remove tube from the magnetic stand and add 600 µl Buffer WB3.
19. Cap the tube and vortex for 10 seconds. Confirm that all beads are resuspended. If beads are still sticking to the wall continue vortexing until all beads are resuspended.
20. Place the tube on the magnetic stand and incubate for 5 minutes until the beads have been pulled to the magnet.
21. Remove and discard as much of the supernatant as possible using a 200 µl pipette. Use a 20 µl pipette to remove the remaining liquid from the bottom of the tube.

> **CAUTION:** 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.

Optional step recommended for FFPE cores: Repeat wash steps 18 through 21. The beads may not fully re-suspend in the 2nd wash with WB3 which does not present a problem.

22. Remove tube from the magnetic stand and add 600 µl 80% ethanol to the tube.
23. Cap the tube and vortex for 10 seconds. Confirm that all beads are resuspended. If beads are still sticking to the wall continue vortexing until all beads are resuspended.
24. Place the tube on the magnetic stand and incubate for 2 minutes until the beads have been pulled to the magnet.
25. Remove and discard the supernatant without disturbing the bead pellet.
26. Remove the tube from the magnetic stand and add 300 µl 80% ethanol.

27. Cap the tube and vortex for 10 seconds. Confirm that all beads are in suspension. If beads are still sticking to the wall continue vortexing until all are suspended.
28. Place the tube on the magnetic stand and incubate for 2 minutes until the beads have been pulled to the magnet.
29. Remove and discard as much of the supernatant as possible using a 200 µl pipette. Use a 20 µl pipette to remove the remaining liquid from the bottom of the tube.
30. Leave the tube open on the magnetic stand and let the beads dry for 6 minutes at room temperature.

> **CAUTION:** Make sure that the ethanol has evaporated before continuing with elution. Residual ethanol can inhibit the elution and impact downstream applications such as PCR.

31. Remove the tube from the magnetic stand and add 50 to 100 µl of Buffer BE (5 mM TrisCl pH 8.5) into the tube.
32. Resuspend the beads by pipetting up and down 20 times. Ensure that all of the beads are resuspended in the buffer and none are still sticking to the wall of the tube.
33. Cap the tube and incubate the microcentrifuge tube in the heat block set to 56 °C for 5 minutes.
34. Remove the tube from the heat block, place it on the magnetic stand, and incubate for 2 minutes.
35. Transfer the eluate into a clean elution tube without transferring beads. A small amount of residual paraffin may be visible in the pipette tip. This will not adversely affect downstream processing of the eluted DNA. Isolated DNA should be kept at 2 to 8 °C for short term storage (1 to 2 days) and -20 °C for long term storage.

Appendix

Appendix A: Optional DNase Treatment of Extraced 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 performed after Step 11 in Section-6 (RNA Purification).

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

1. Prepare a 1 X TURBO DNase master mix:

Reagent	Volume for one sample*	Volume for N samples*
RNase-free H2O	93.5 µl	93.5 µl x N
10X TURBO DNase buffer	11 µl	11 µl x N
TURBO DNase	5.5 µl	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. Resuspend the beads by pipetting up and down 20 times.
4. Incubate at room temperature for 30 minutes.
5. Add 300 µl of BB3 and vortex for 5 seconds.
6. Incubate at room temperature for 10 minutes.
7. Place the tube on a magnetic stand and incubate for 5 minutes until the beads have been pulled to the magnet.
8. Carefully remove and discard the supernatant using a 200 µl pipette. Avoid disturbing the bead pellet.
9. Remove the tube from the magnetic stand and add 600 µl WB4 to the tube.
10. Cap it and vortex thoroughly until all beads are resuspended.
11. Place the tube back on the magnetic stand and incubate for 5 minutes until the beads have been pulled to the magnet.
12. Remove as much of the supernatant as possible using a 200 µl pipette. Use a 20 µl pipette to remove the remaining liquid from the bottom of the tube.
13. Proceed with Step 12 in Section-6 (RNA Purification).

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Appendix B: Troubleshooting Guide

Issue	Cause	Solution	Comments / Suggestions
Low DNA yield	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 56C, 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.	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 pipette 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 RNA yield	Loss of magnetic beads during purification steps.	Remove supernatant of bind and wash steps slowly and carefully. If beads appear in the pipette tip, eject the liquid back into the tube, wait for 1 minute, and try aspirating the supernatant again.	See sample input guidelines in Section-1.
	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.	The viscosity of buffers BB3, WB3 and WB4, as well as the presence of the paraffin emulsion can make supernatant removal difficult.
	Insufficient tissue input.	Select FFPE section with higher tissue to wax ratio or add additional section.	In your initial use of the truXTRAC FFPE total NA kit use FFPE blocks that have been well characterized for yield and quality.
Beads clumpy during elution and DNA and/or RNA yield low	Residual paraffin in elution.	For samples with a high paraffin content, a second wash buffer wash may be required to completely remove the paraffin.	If the paraffin emulsion was not completely removed in the wash steps, residual wax can be carried through to the elution step.
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.

Tips for Determining Quality and Quantity of the Purified FFPE 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 can be used to assess the amplifiability of isolated DNA as well as the presence of inhibitors. Note that DNA from FFPE tissue itself can act as inhibitor at high input concentrations due to the extensive damage (nicks, depurination, etc.). Therefore, a dilution series over at least 5 orders of magnitude starting with undiluted material of the extracted DNA should always be done when assessing quality by qPCR. An example is shown in Dietrich et al. Figure 1 [3].

Additional Notes

1. See following link: 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. 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