Covaris®

# truXTRAC® FFPE RNA Plus Kit – Magnetic Beads

Adaptive Focused Acoustics® (AFA®)-based RNA extraction from FFPE tissues using magnetic bead-based purification

PN 520271



Part Number: 010482 Rev C | July 2020

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# General Information

## Intended Use

The truXTRAC FFPE RNA Plus Kit – Magnetic Beads is intended for research use only. This product is not intended for the diagnosis, prevention, or treatment of any disease.

## Introduction

The truXTRAC FFPE RNA Plus Kit is designed for efficient and sequential extraction of RNA 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 for sensitive analytical methods such as next-generation sequencing (NGS) or RT-qPCR. Finally, the workflow is simple in that it does not require any centrifugation steps.

This protocol is optimized for up to 20 µm of total FFPE tissue sections or two FFPE cores (1.2 mm in diameter and 5 mm in length).

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

***Important Notes on FFPE Samples:***

The yield of RNA from FFPE tissue blocks is highly variable. Factors such as fixation time, 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 RNA isolated from FFPE samples can also be highly variable. During the fixation process, RNA is 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 [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.

## Revision History

| Part Number | Revision | Date | Description of Change |
|---|---|---|---|
| 010482 | A | 10/19 | Initial Release of truXTRAC FFPE RNA Plus Kit – Magnetic Beads |
| 010482 | B | 10/19 | Updated RNA purification protocol |
| 010482 | C | 7/20 | Update to required reagents |

## Kit Contents

- RNA Lysis Buffer ..... 13 ml
- Proteinase K (PK Solution)..... 1.3 ml
- Magnetic Bead Suspension ..... 0.25 ml
- Buffer BB3..... 24 ml
- Buffer WB3 ..... 50 ml
- RNA Elution Buffer ..... 3 ml
- microTUBE-500 AFA Fiber Screw-Cap FFPE ..... 24

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

## Storage

Upon kit arrival, store the PK 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
- Dry block heater for 2 ml tubes or temperature-controlled water bath able to accurately heat between 50 to 90 °C
- Magnet Stand for 2 ml tubes (e.g. Thermo Fisher Scientific, DynaMag-2 Magnet, PN 12321D)

### Required Reagents

- 100% ethanol, molecular biology grade (e.g., AmericanBio, PN AB00515)
- Nuclease-free water (e.g., Invitrogen, PN AM9930)
- TURBO™ DNase (Thermo Fisher Scientific, PN AM2238), containing 10X TURBO DNase buffer

### 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.

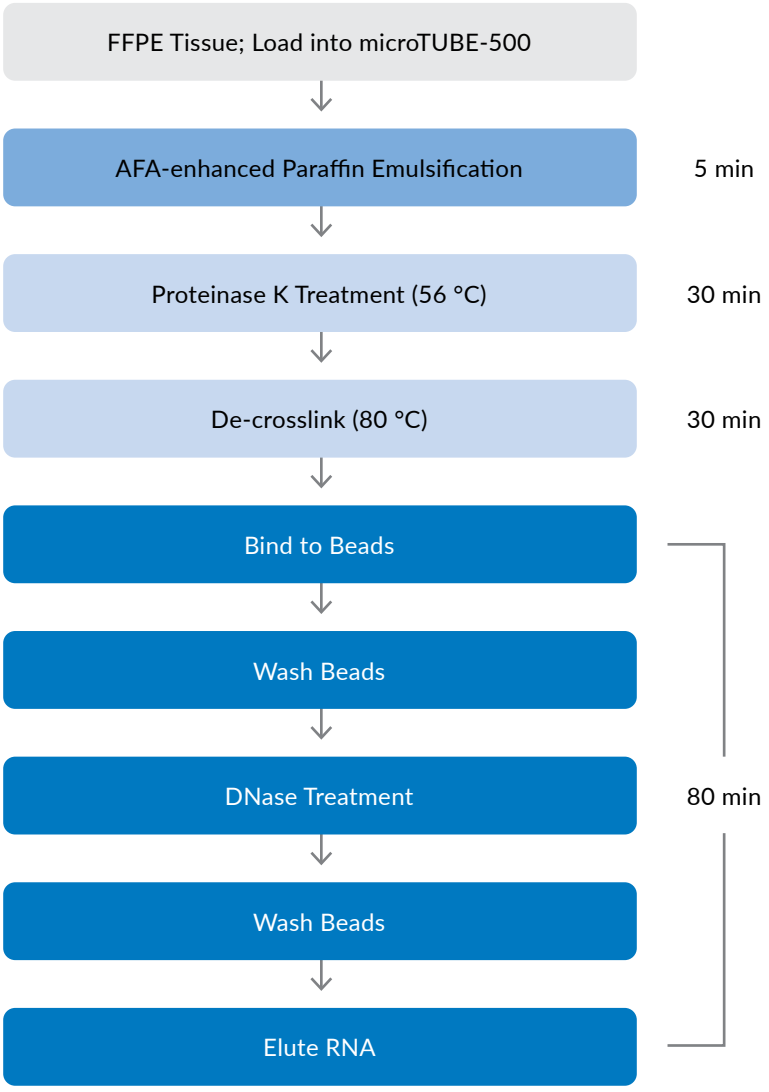
| Instrument | M220 | ME220 | S220 | E220*evolution* | E220 | LE220/LE220-plus* |
|---|---|---|---|---|---|---|
| Holder/Rack Description (PN) | Holder XTU (500414) | Rack 4 Position microTUBE-500 (500525) | Holder microTUBE-500 Screw Cap (500449) | Rack E220e 4 microTUBE-500 Screw Cap (500484) | Rack 24 microTUBE-500 Screw Cap (500452) | Rack 24 microTUBE-500 Screw Cap (500452) |
| Plate Definition File Name | N/A | <4 microTUBE-500 Screw-Cap PN 520185> | N/A | <500484 E220e 4 microTUBE-500 Screw-Cap> | <500452 Rack 24 Place microTUBE-500 Screw-Cap> | <500452-24 microTUBE-500 Screw-Cap +6mm offset z enabled> |
| Required Accessories (PN) | Insert XTU (500471) | ME220 Waveguide 4 Place (500534) | N/A | Intensifier (500141) | Intensifier (500141) | N/A |

*Please find the LE220 and LE220-plus plate definition files here: https://covaris.com/resources/registered-users-login/l-series/

## FFPE RNA Extraction and Purification Workflow

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

Using the Adaptive Focused Acoustics (AFA) process, FFPE samples are prepared in RNA Lysis Buffer in the presence of Proteinase K. RNA is released from the tissue by AFA-enhanced Proteinase K digestion, followed by a de-crosslinking step. RNA is then purified using magnetic beads, which includes a DNase treatment step.



## 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:**

Scalpel or razor blade collection

| Slide Collection Method | Maximum Input per microTUBE-500 |
|---|---|
| Scalpel or razor blade to scrape material from slides | 20 µm of total thickness<br>(e.g., 4 slides at 5 µm thick = 20 µm total thickness)<br>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. We recommend no more than 1-part wax to 2-part tissue.

| FFPE Curl/Scroll Thickness | Maximum Scrolls per microTUBE-500 |
|---|---|
| 5 µm | 4 |
| 10 µm | 2 |
| 15 µm | 1 |

**FFPE Core Input Requirements**:

For best results, minimize the amount of wax present by trimming. After punching the core from the block, use a razor blade to remove any edges with paraffin.

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

## 2 - Preparation of Reagents

***Note***: Follow these instructions before starting the FFPE RNA isolation protocol.

1. **RNA Lysis Buffer**: Visually check for a white precipitate that may form during storage before each use. If white precipitate is visible, incubate the RNA Lysis Buffer at 50 to 60 °C for 5 to 10 minutes before use to dissolve any precipitate.
2. **80% Ethanol Solution**: Prepare the 80% ethanol solution by mixing 4 parts 100% ethanol with 1 part nuclease-free water. One sample requires **1.3 ml** of 80% ethanol. To prepare the total amount of 80% ethanol needed, multiply the number of samples to be processed by **1.5 ml** to account for dead volume.

## 3 - Preparation of Heat Blocks

1. Preheat dry block heaters to 37 °C, 56 °C, and 80 °C ± 2 °C. It is critical that these temperatures are accurate to successfully execute the protocol.
2. To test the temperature of your heat blocks for use with the microTUBE-500:
    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 ± 2 °C.

> ***CAUTION:*** The microTUBE-500 must be used in conjunction with the 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. 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

**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.

Refer to ***Appendix A*** for instructions on adding dithering parameters to the method.

| Instrument | M220 | ME220 | S220 | E220*evolution* | E220 | LE220 / LE220-plus |
|---|---|---|---|---|---|---|
| **Peak Incident Power (PIP) (Watt)** | 75 | 75 | 200 | 200 | 200 | 450 |
| **Duty Factor (%)** | 25 | 25 | 10 | 10 | 10 | 20 |
| **Cycles Per Burst (CPB)** | 200 | 1000 | 200 | 200 | 200 | 200 |
| **Treatment time (seconds)** | 360 | 300 | 300 | 300 | 300 | 300 |
| **Bath temperature (C)** | 20 | 20 | 20 | 20 | 20 | 20 |
| **Dithering (See Appendix A for dithering setup)** | N/A | Z Dither: 2 mm Dither Speed: 5 mm/sec | N/A | N/A | N/A | Z Dither: 3 mm Dither Speed: 5 mm/sec |
| **Water Level (run)** | Full | Auto | 8 | 6 | 6 | 6 |

## 5 - Paraffin Emulsification, Tissue Rehydration, and Lysis

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

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

> ⚠ ***CAUTION:*** The RNA Lysis Buffer/Proteinase K Solution Mix should be stored at ambient temperature and used within 30 min of preparation.

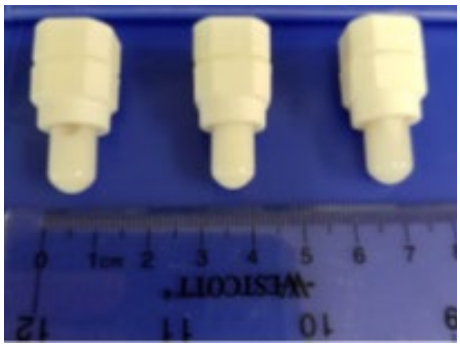
| Reagent | Volume for one sample* | Volume for N samples* |
|---|---|---|
| RNA Lysis Buffer | 440 µl | 440 µl x N |
| Proteinase K Solution | 44 µl | 44 µl x N |

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

2. Load each FFPE sample into a microTUBE-500.
3. Add **440 µl** of the RNA Lysis Buffer/Proteinase K 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 sample 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 on the heat block set to 56 °C.
8. Incubate for 30 minutes at 56 °C.

> ***CAUTION:*** It is recommended to increase the incubation time to 2 hours for core punch samples.

9. Remove the microTUBE-500s with the adapters from the heat block and transfer directly to dry heat block set for 80 °C incubation.
10. Incubate for 30 minutes at 80 °C.
11. Remove the microTUBE-500s 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.

## 6 - RNA Purification

1. Verify that two heat blocks are set to 37 °C (Step 15) and 56 °C (Steps 5 and 35).
2. Prepare the BB3/Magnetic Bead Mix according to ***Table 2***.

| Reagent | Volume for one sample* | Volume for N samples* |
|---|---|---|
| BB3 | 607 µl | 607 µl x N |
| Magnetic Bead Suspension | 8.8 µl | 8.8 µl x N |

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

> ***CAUTION:*** 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.

3. Add **560 µl** of the BB3/Magnetic Bead Mix to the RNA solution in the 2 ml microcentrifuge tube.
4. Cap the microcentrifuge tubes and vortex for 10 seconds.
5. Incubate the microcentrifuge tubes at 56 °C for 5 minutes.
6. Place the microcentrifuge tubes on a magnetic stand and wait for 5 minutes or 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.

7. With the microcentrifuge tubes still on the magnet, carefully remove and discard the supernatant. Avoid disturbing the bead pellet.
8. Remove the microcentrifuge tubes from the magnetic stand and add **1 ml** Buffer WB3.
9. Cap the microcentrifuge tubes and vortex for 10 seconds. Confirm that all beads are resuspended. If beads are still sticking to the wall continue vortexing until all are resuspended.
10. Place the microcentrifuge tubes back on the magnet stand and wait for 5 minutes, until the beads have been pulled to the magnet.
11. With the microcentrifuge tubes on the magnet, carefully remove, and discard the supernatant.

## 6 - RNA Purification (cont.)

12. Prepare the 1X TURBO DNase Treatment Mix according to ***Table 3***.

| Reagent | Volume for one sample* | Volume for N samples* |
|---|---|---|
| Nuclease-free $H_2O$ | 93.5 µl | 96.8 µl x N |
| 10X TURBO DNase buffer | 11 µl | 11 µl x N |
| TURBO DNase | 5.5 µl | 2.2 µl x N |

**Table 3**. DNase Treatment Mix
*Calculation includes 10% excess in final volume.

> ⚠ ***CAUTION:*** Follow enzyme best practices when working with DNase. Prepare the DNase Treatment Mix immediately before use. Mix the DNase Treatment Mix gently.

13. Add **100 µl** of DNase Treatment Mix to each bead pellet.
14. Re-suspend the beads by pipetting up and down 20 times.
15. Incubate at 37 °C or ambient temperature for 30 minutes.
    ***Note***: DNase treatment at 37 °C will result in more efficient DNA removal.
16. Add **200 µl** of Buffer BB3 and vortex for 5 seconds.
17. Place the microcentrifuge tubes on a magnetic stand and incubate for 5 minutes or until the beads have been pulled to the magnet.
18. Carefully remove the supernatant using a 200 µl pipet. Avoid disturbing the bead pellet.
19. Remove the microcentrifuge tubes from the magnetic stand and add **1 ml** Buffer WB3.
20. Cap the microcentrifuge tubes and vortex for 10 seconds. Confirm that all beads are resuspended. If beads are still sticking to the wall continue vortexing until all are resuspended.
21. Place the microcentrifuge tubes back on the magnet stand and wait for 5 minutes, until the beads have been pulled to the magnet.
22. With the microcentrifuge tubes on the magnet, carefully remove and discard the supernatant.
23. After the final wash, remove as much of the supernatant as possible. Use a 20 µl pipettor to remove the remaining liquid from the bottom of the tubes.

> ⚠ ***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.

24. Remove the microcentrifuge tubes from the magnetic stand and add **1 ml** 80% ethanol solution to the tubes.
25. Cap the microcentrifuge tubes and vortex for 10 seconds. Confirm that all beads are resuspended. If beads are still sticking to the wall continue vortexing until all are resuspended.

## 6 - RNA Purification (cont.)

26. Place the microcentrifuge tubes back on the magnet stand and wait for 2 minutes, until the beads have been pulled to the magnet.
27. With the microcentrifuge tubes on the magnet, carefully remove and discard the supernatant.
28. Remove the microcentrifuge tubes from the magnetic stand and add **300 µl** 80% ethanol solution.
29. Cap the microcentrifuge tubes and vortex for 10 seconds. Confirm that all beads are resuspended.
30. Place the microcentrifuge tubes on the magnetic stand and wait for 2 minutes, 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 the 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.

> ***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.

33. Remove the microcentrifuge tubes from the magnetic stand and add **50 to 100 µl** of RNA Elution Buffer 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, 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 RNA.
38. Store the eluted RNA on ice until further processing. Isolated RNA should be kept at −80 °C for long-term storage.

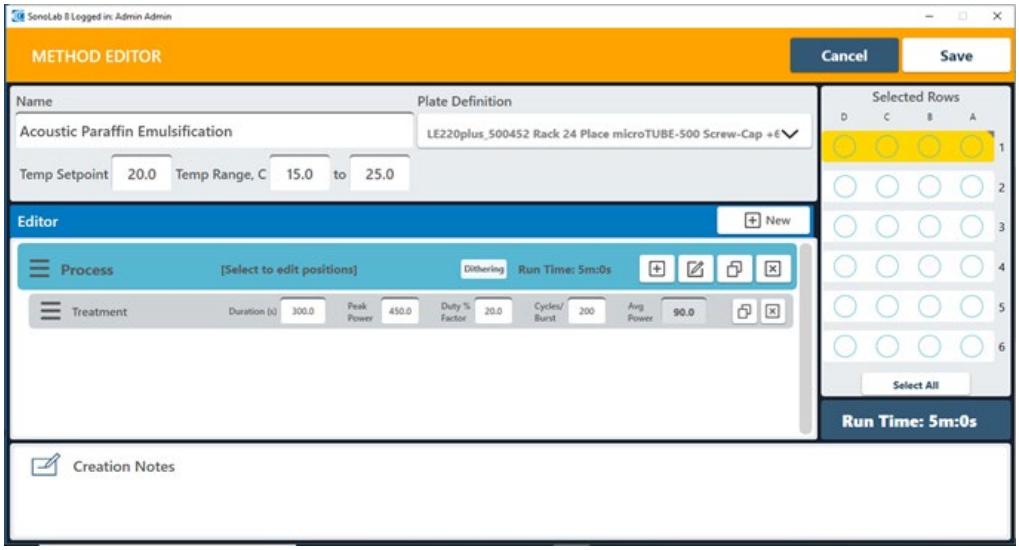
# Appendix

## Appendix A - Protocol Creation in SonoLab

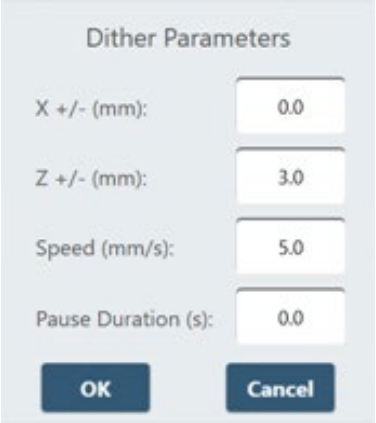
**LE220-plus with SonoLab 8.4 or higher**

Refer to the LE220-plus Manual: www.covaris.com/wp-content/uploads/pn_010398.pdf

1. On the Home screen, click the “New” button to bring up the Method Editor.
2. In the Method Editor, set up the new method as pictured below.




3. For Dithering: Click the “Dither Parameter” button in the Process Editor, input the X, Z, and Speed dithering values shown below. Then click the “OK“ button.



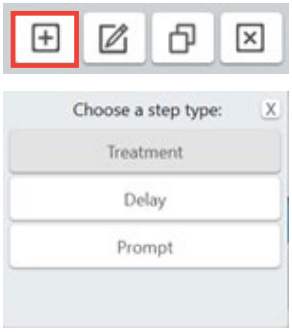

4. Edit the remaining parameters to match the Method Editor screenshot above.
5. Select the sample rows to run.
6. Click the “Save” button once finished.

## Appendix A - Protocol Creation in SonoLab (cont.)

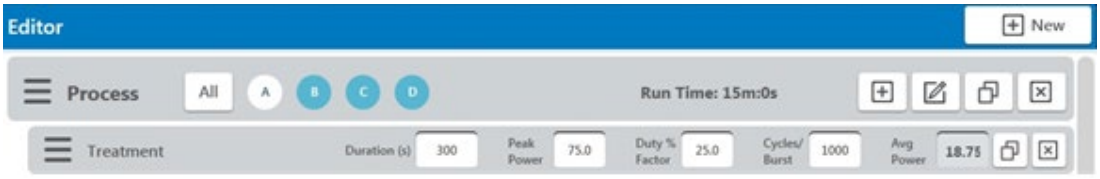
**ME220 with Sonolab 8**

Refer to the ME220 Manual: www.covaris.com/wp-content/uploads/pn_010325.pdf

1. Click the "New" button from the main screen.
2. Input the protocol name as "*Acoustic Paraffin Emulsification*".
3. Select the **<4 microTUBE-500 Screw-Cap PN 520185>** Rack Definition from the drop-down menu.
4. Click the "+ New Item" (Add New Process or Repeat) button.
5. In the Process Bar click "Add a step to the process" and then select "Treatment".

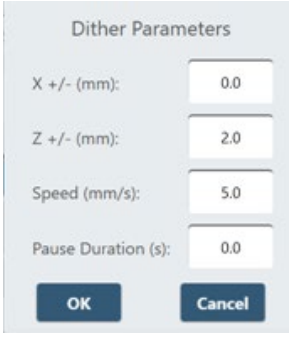



6. Change the AFA settings in the Treatment bar as shown below.




7. Click the "Edit Process Settings" button.

8. Input the X- and Z-dithering values and then click "OK".




9. In the Editor box, select the number of samples to run in the Process bar.
10. Click the "Save" button once finished.

## Appendix B - Pre-treatment for Mineral Oil Stabilized FFPE (Optional)

### Introduction

The Pre-Treatment for Mineral Oil-Stabilized FFPE is specifically designed to prepare FFPE tissue samples such as scrolls, or slide scrapings that are submerged in mineral oil for downstream extraction of nucleic acids. Once a 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).

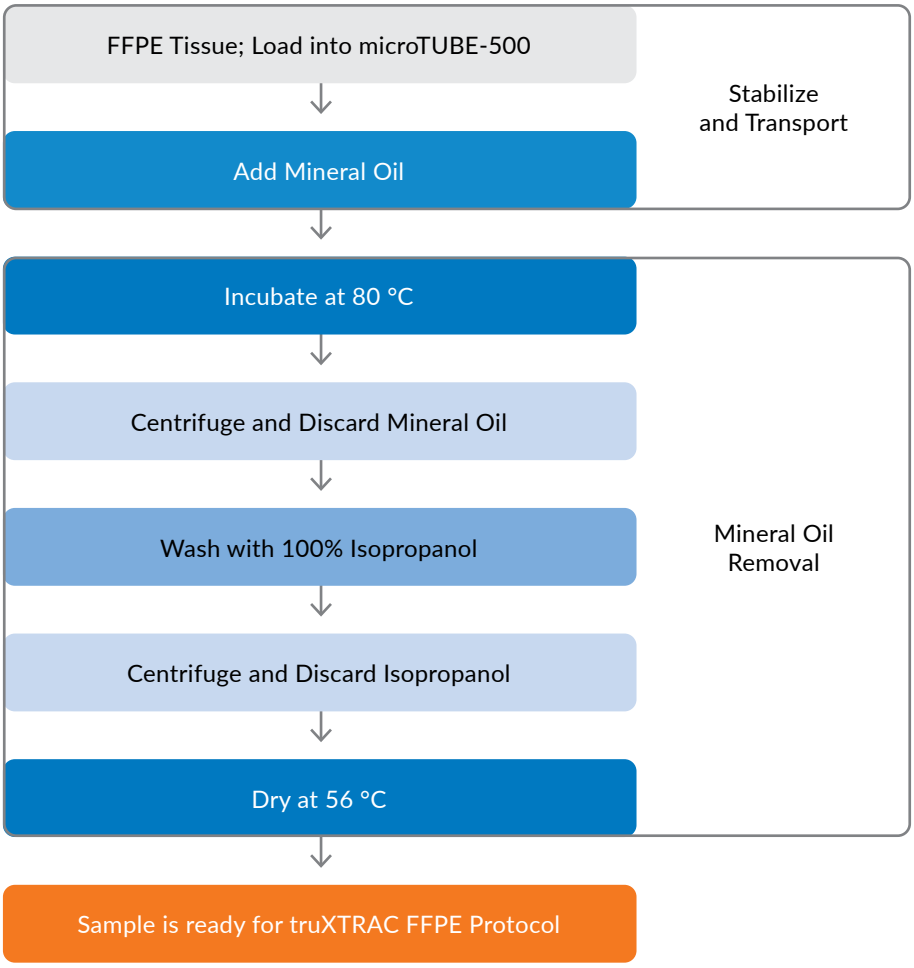
Note that the FFPE sample input requirements are different than the standard, no mineral oil preserved samples. The maximal amount of tissue per microTUBE increases with the addition of mineral oil.

However, the mineral oil treatment may reduce RNA recovery in samples with low input amounts. Please refer to the sample input tables on page 18.

### 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



## 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

| Scalpel or Razor Blade Collection | | |
|---|---|---|
| **Slide Collection Method** | **Minimum Input** | **Maximum Input** |
| Scalpel or razor blade to scrape material from slides | 15 µm of total sample thickness<br>(e.g., 3 slides at 5 µm thick = 15 µm total thickness)<br>Min Area (on each slide): 7.5 mm x 7.5 mm | 40 µm of total sample thickness<br>(e.g., 8 slides at 5 µm thick = 40 µm total thickness)<br>Max Area (on each slide): 15 mm x 15 mm |

| Curls/Scrolls Input Requirements | | |
|---|---|---|
| **FFPE Curls/Scrolls Thickness** | **Minimum Input** | **Maximum Input** |
| 5 µm | 2 | 8 |
| 10 µm | 1 | 4 |
| 15 µm | 1 | 2 |

| Core Punch Input Requirements | | |
|---|---|---|
| **FFPE Core Punch Diameter** | **Minimum Input** | **Maximum Input** |
| ≤ 1.2 mm; 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) ± 2 °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.
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:*** When removing the supernatant in the microTUBE-500, it is critical to not disturb the tissue. Depending on the tissue collection and input amount, the tissue could be a small pellet or large pieces of tissue.
>
> a. Locate the RNA-containing tissue pellet. It will be located on the same side as the barcode which faces outward during centrifugation.
> b. Slightly tilt the tube away from the tissue.
> c. Using a 200 µl pipette tip, slowly and carefully 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**.

7. Add **400 µl** of 100% isopropanol.
8. Vortex briefly for 3 seconds.

## Removal of Mineral Oil (cont.)

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 the 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 (Paraffin Emulsification, Tissue Rehydration, & Lysis).

## Appendix C - Troubleshooting Guide

| Issue | Cause | Solution | Comments / Suggestions |
|---|---|---|---|
| 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 pipet tip, eject the liquid back into the tube, wait for 1 minute, and try aspirating the supernatant again. | The viscosity of buffers BB3 and WB3, 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. |
| 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. |
| Bead clumping during elution | Residual paraffin in elution. | Lower the amount of paraffin in the sample by trimming paraffin off the FFPE tissue block. | Too much paraffin in the input samples(s) may not completely be removed during the purification. |

## Tips for Determining Quality and Quantity of the Purified FFPE RNA

- To determine RNA yields, a fluorometric assay such as Qubit™ (Life Technologies) should be used.
- In addition, spectrophotometric analysis determining the A260/280 and A260/230 ratios will determine if protein or peptide/salt contamination is present in the sample.
- RT-qPCR can be used to assess the amplifiability of isolated RNA as well as the presence of inhibitors. Note that RNA 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 RNA should always be done when assessing quality by RT-qPCR. An example is shown in Dietrich et al. ***Figure 1*** [3].

## Additional Notes

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

## References


1. Carrick et al. (2015) Robustness of Next Generation Sequencing on Older Formalin-Fixed Paraffin-Embedded Tissue. PLoS ONE 10(7): e0127353.
2. 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.