Covaris®

truXTRAC® FFPE Total NA 96 Manual Kit

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

Manual Version for up to 96 Samples (PN 520316 & PN 500731)

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Intended Use

The truXTRAC FFPE Total NA Auto 96 Manual Kit and truXTRAC FFPE Total NA Auto 96 Manual Accessory Kit are 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 Auto 96 Manual Kit and truXTRAC FFPE Total NA Auto 96 Manual Accessory Kit are 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) in a fully automated workflow.

The truXTRAC FFPE Total NA Auto 96 Manual Kit and truXTRAC FFPE Total NA Auto 96 Manual Accessory Kit are specifically designed and written to allow a manual evaluation for demo purposes. The workflow follows the automated version closely and relies on many accessories/instrumentations that are required for the fully automated version.

The first step in the workflow represents excess paraffin removal using Deparaffinization Solution. In the following step, after addition of Tissue Lysis Buffer, AFA-energetics® enables tissue rehydration, homogenization, and simultaneous active removal of residual paraffin from FFPE tissue samples. AFA-enhanced tissue homogenization is highly efficient for extracting macro-molecules such as nucleic acids from FFPE samples. AFA in combination with a mild aqueous buffer results in increased nucleic acid yields while minimizing degradation of nucleic acids exposed at the FFPE section surface. Thus, the truXTRAC protocol results in high yields of high-quality RNA and DNA, ready for sensitive analytical methods such as next-generation sequencing (NGS) or qPCR/RT-qPCR.

The fully automated protocol is optimized for a sample input of one 10 µm FFPE tissue section per tube and configured for AFA-treatment of 96 samples in parallel, following 96 well-based purification in a semi-automated workflow.

This protocol is written for manual processing of up to 16 samples in parallel. It involves most of the instrumentation and accessories required for fully automated processing but incorporates manual pipetting and transfer steps, as well as manual movement of the sample tubes from one processing station to the next.

Important Notes on FFPE Samples:

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

More importantly, the quality of DNA and RNA isolated from FFPE samples can be highly variable. During the fixation process, DNA and RNA are cross-linked to proteins and among nucleic acids 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, check the FAQ's found on our website, or contact Covaris Application Support at applicationsupport@covaris.com.

Important Notices

1. **Incubation during Proteinase K treatment and de-crosslinking:** Incubation times and temperatures during Proteinase K digestion (at 56 °C) and de-crosslinking (at 80 °C) are very critical for successful extraction and purification of nucleic acids from FFPE. You must follow the recommended process to check and adjust temperatures on the heat block to ensure proper temperatures in the reaction tubes. This may require that the heat blocks be set to a higher temperature than listed to ensure that the sample reaches the appropriate temperature.
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 a centrifugation step. At this critical step in the protocol, RNA is concentrated 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. Incomplete separation of fractions can result in decreased yield and loss in quality.
3. **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 this impact greatly. However, the user must follow the recommendations (see ***Page 7***) for sample input.

Kit Contents

- Deparaffinization Solution* ..... 68 mL
- Rehydration Buffer ..... 13 mL
- Tissue Lysis Buffer ..... 65 mL
- Proteinase K Solution ..... 11 mL
- Magnetic Bead Suspension ..... 4 mL
- Buffer BB2 ..... 58 mL
- Buffer WB5 ..... 300 mL
- RNA Elution Buffer ..... 15 mL
- Buffer BE ..... 15 mL
- truTUBE Screw-Cap 96
- 16 Home 1
- 96 Shuttle 1
- RFID Labels 5

Accessory Kit Contents

- PSU Rack R&L-Series 96 Shuttle (PN 500732) 1
- Dry Block Heater 96 Shuttle 1
- Heat Block Manual 96 Shuttle 1
- Dry Block Heater 16 Microcentrifuge Tube 2
- Heat Block Manual Microcentrifuge Tube 2
- Stand 1.5 mL Tubes Magnetic 1
- Decapper truTUBE Screw-Cap (PN 350402) 1
- Shuttle Weight (PN 500795) 1

*The Deparaffinization Solution is not compatible with Polystyrene labware. Many reagent reservoirs for automation are made from polystyrene.

Storage Requirements

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

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

Laboratory Equipment, Chemicals, and Consumables Supplied by User

Required Laboratory Equipment and Accessories

- Benchtop centrifuge with swinging bucket rotor for plates (5,000 x g attainable)
- Benchtop centrifuge with rotor for 1.5 mL tubes
- 1.5 mL microcentrifuge tubes (e.g., Eppendorf® Safe-Lock Tubes, PN 022363204)

Required Chemical and Enzymes

- TURBO™ DNase with 10x buffer (Thermo Fisher Scientific®, PN AM2238)
- 100% Ethanol, molecular biology grade (e.g., Millipore-Sigma®, PN E7023)
- 100% Isopropanol, molecular biology grade (e.g., Millipore-Sigma, PN I9516)
- Nuclease-free water (e.g., Invitrogen™, PN AM9930)
- Optional DNase-free RNase A (10 mg/mL) (e.g., Thermo Fisher Scientific, PN EN0531)

Focused-ultrasonicator Accessories and Plate Definitions

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

LE220-plus Focused-ultrasonicator (PN 500569)

Instrument	LE220-plus Focused-ultrasonicator (PN 500569) - SonoLab 8.5 and SonoLab 10.2
Rack Description	PSU Rack R&L-Series 96 Shuttle (PN 500732)
Accessory	Shuttle Weight (PN 500795)
Plate Definition File Name	Contact applicationsupport@covaris.com

LE220R-plus Focused-ultrasonicator (PN 500578)

Instrument	LE220R-plus Focused-ultrasonicator (PN 500578) - SonoLab 8.5 and SonoLab 10.2
Rack Description	PSU Rack R&L-Series 96 Shuttle (PN 500732)
Accessory	Shuttle Weight (PN 500795)
Plate Definition File Name	Contact applicationsupport@covaris.com

LE220Rsc Focused-ultrasonicator (PN 500652)

Instrument	LE220Rsc Focused-ultrasonicator (PN 500652) - SonoLab 8.5 and SonoLab 10.2
Rack Description	PSU Rack R&L-Series 96 Shuttle (PN 500732)
Accessory	Shuttle Weight (PN 500795)
Plate Definition File Name	Contact applicationsupport@covaris.com

R230 Focused-ultrasonicator (PN 500620)

Instrument	R230 Focused-ultrasonicator (PN 500620) – SonoLab 10.0.0 or higher
Rack Description	PSU Rack R&L-Series 96 Shuttle (PN 500732)
Accessory	Shuttle Weight (PN 500795)
Plate Definition File Name	Contact applicationsupport@covaris.com

FFPE Deparaffinization, tNA Extraction, and Purification Workflow

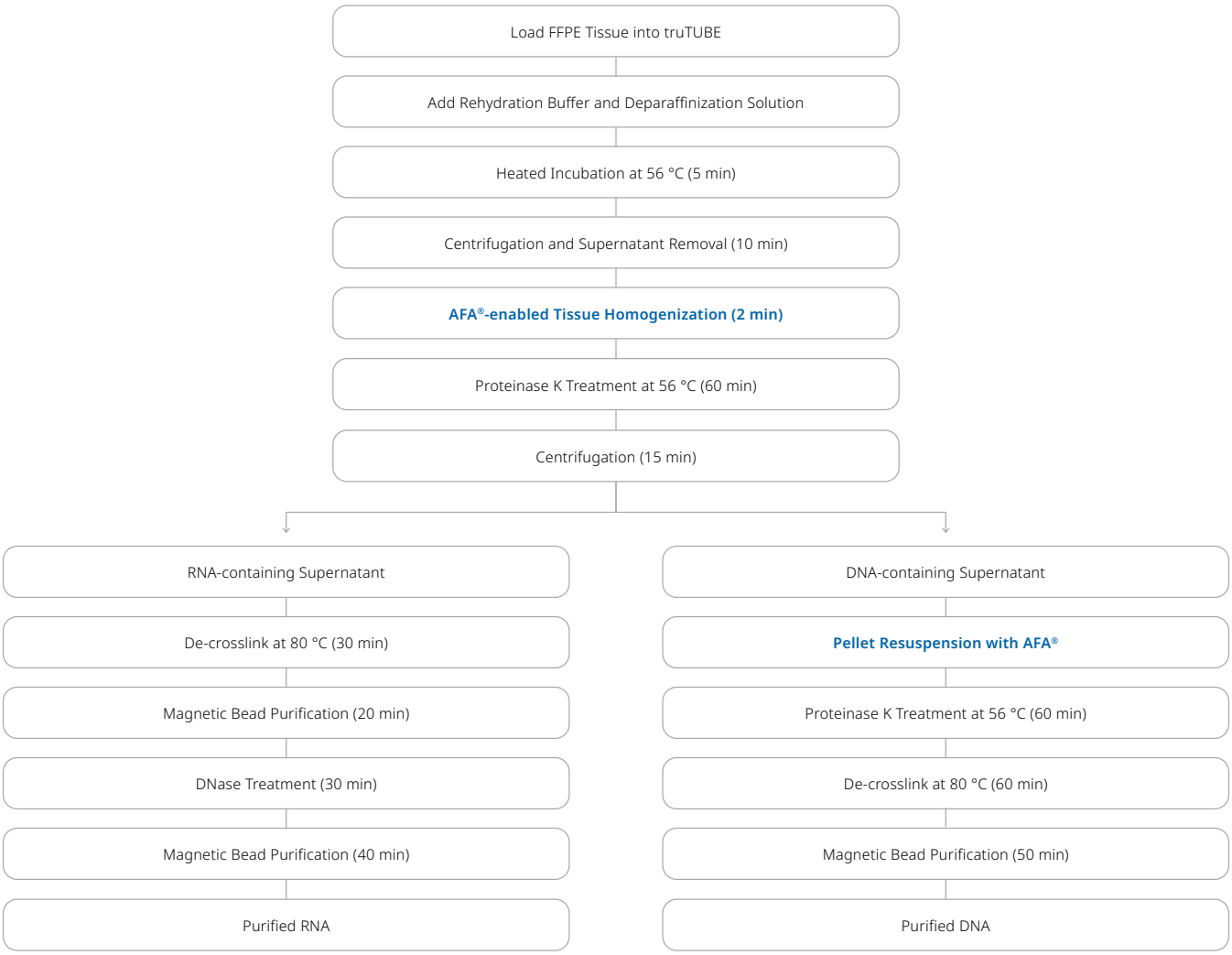
After loading the 10 µm section into the truTUBE, the tissue is rehydrated by adding Rehydration Buffer. The Rehydration Buffer can be added ***BEFORE*** loading the sample if required.

With the addition of the Deparaffinization Solution and heating of the truTUBE to 56 °C, a major part of the paraffin is dissolved. The Deparaffinization Solution and the dissolved paraffin are separated from the sample by a centrifugation step and is subsequently removed by pipetting.

The Tissue Lysis Buffer and Proteinase K are now added to the tissue, following treatment with AFA to fully rehydrate, homogenize the tissue, and to 'scrub' it clean from any residual paraffin. During the following Incubation at 56 °C, RNA is released while genomic DNA remains in the tissue.

The RNA-containing supernatant is separated from the DNA-containing tissue by a centrifugation step. RNA is then de-crosslinked and purified following a magnetic bead-based workflow.

The tissue pellet containing the DNA is resuspended in Tissue Lysis Buffer and Proteinase K by applying AFA, followed by a Proteinase K treatment to release the DNA. After de-crosslinking incubation the DNA is purified following a magnetic bead-based workflow.



FFPE Sample Input Requirements and Guidelines

Curls/Scrolls Input Requirements: Total thickness of 10 µm, non-trimmed FFPE section

Slide Section Input Requirements: Total thickness of 10 µm (e.g., two slide scraping of 5 µm thickness each)

Working with Deparaffinized Slides:

An optional shortened version of the protocol can be followed when processing deparaffinized slides. This shortened workflow omits the 'Paraffin Removal' section.

1. Add 2 µL of Tissue Lysis Buffer on the slide directly near the tissue to help soften the tissue and facilitate removal.
2. Use a razor or scalpel to collect the tissue. Transfer into the bottom of the truTUBE Screw-Cap and into the Rehydration Buffer.
3. Continue with the 'Tissue Homogenization' section.

> **NOTE:** The full unmodified workflow can be used with deparaffinized slide samples, for example, if combining paraffinized and deparaffinized samples in the same plate/sample set.

1 - Preparation of Reagents

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

1. **Tissue Lysis Buffer and Buffer BB2**: Visually check for a white precipitate that may form during storage before each use. If a precipitate is visible, incubate the buffer bottle at 50–60 °C for 5–10 minutes before use to dissolve any precipitate. Mix the buffer by inverting the bottle 10 times before using it.
2. **Buffer WB5**: This buffer is supplied as a concentrate. Before use, the buffer needs to be diluted with 100% Isopropanol. Mix 36 mL of WB5 and 24 mL of 100% Isopropanol in a separate bottle. Use the diluted WB5 for all WB5 Wash Steps. Alternatively, add 200 mL of 100% Isopropanol directly to the 300 mL bottle of WB5 Concentrate and mark the bottle to indicate this.
3. **80% Ethanol**: Prepare the 80% ethanol solution by mixing 4 parts 100% ethanol with 1 part nuclease-free water. For 16 samples, mix 40 mL 100% ethanol with 10 mL nuclease free water to obtain 50 mL of 80% ethanol.

2 - Preparation of Heat Blocks

1. Preheat the Dry Block Heater 96 Shuttle equipped with the Heat Block Manual 96 Shuttle to 56 ºC and measure temperature in a truTUBE with water. Allow the temperature to equilibrate and adjust the heater until the final temperature is 56 °C ± 2 ºC within the vessel.
2. Preheat one Dry Block Heater 16 Microcentrifuge Tube equipped with Heat Block Manual Microcentrifuge Tube to 56°C ± 2 ºC and measure temperature in the 1.5 mL tube with water. Allow the temperature to equilibrate and adjust the heater until the final temperature is 56 °C ± 2 ºC within the vessel.

> **NOTE:** Dry Block Heater16 Microcentrifuge equipped with Heat Block Manual Microcentrifuge Tube may require > 5 °C offset to reach correct temperature in the vessel.

3. Preheat the second Dry Block Heater 16 Microcentrifuge Tube equipped with Heat Block Manual Microcentrifuge Tube to 80 °C and measure temperature in the 1.5 mL tube with water. Allow the temperature to equilibrate and adjust the heater until the final temperature is 80 °C ± 2 °C within the vessel.

3 - Focused-ultrasonicator Programming of the L-Series and R230 Instruments

For detailed instructions on how to prepare and use your L-Series or R230 Instrument, please refer to the respective Covaris User Manual. If you do not see a Plate Definition, please contact Covaris Applications Support (applicationsupport@covaris.com).

> **NOTE:** See ***Appendix B*** PSU Rack R&L-Series 96 Shuttle for more information on inserting the rack into your instrument and on using the Shuttle Weight.

1. **Create "Acoustic Tissue Homogenization" program in SonoLab**: Use the settings provided in the table below to create the "Acoustic Tissue Homogenization" program using the Covaris SonoLab method editor. Save the program.

	L-Series	R230
Peak Incident Power (PIP) (Watt)	300	300
Duty Factor (%)	25	25
Cycles Per Burst (CPB)	600	300
Treatment Time (seconds)	120	120
Bath Temperature (°C)	20	20
Dithering	z Dither: 2 mm; Speed: 5 mm/sec	No Dither

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

	L-Series	R230
Peak Incident Power (PIP) (Watt)	300	300
Duty Factor (%)	25	25
Cycles Per Burst (CPB)	800	300
Treatment Time (seconds)	20	20
Bath Temperature (°C)	20	20
Dithering	No Dither	No Dither

4 - Excess Paraffin Removal

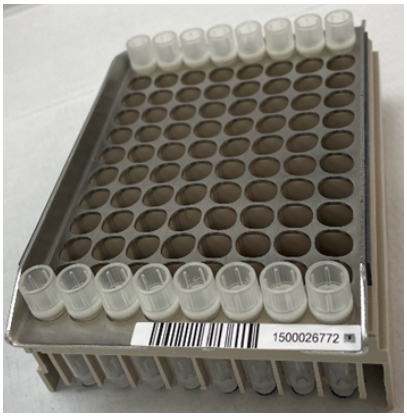
1. Load each FFPE sample into a truTUBE and close with the Screw-Cap or continue with ***Step 2***. Do not use stickers to label the outside of the tubes.

> **NOTE:** For information on using the Decapper truTUBE Screw-Cap tool, see ***Appendix C***.

2. Open the screw cap and add 50 µL Rehydration Buffer to the truTUBE.

> **NOTE:** The Deparaffinization Solution is not compatible with polystyrene labware. Many reagent reservoirs for automation are made from polystyrene.

3. Continue to add 520 µL Deparaffinization Solution and cap the truTUBE. ***DO NOT VORTEX THE TUBE OR MIX THE CONTENTS***.
4. Insert the truTUBEs into the front and back row of the 96 Shuttle sitting on the 16 Home by firmly pressing them down until stop (see picture to right). The tubes should be visible at the very front and back of the 16 Home. ***This configuration (96 Shuttle on the 16 Home with inserted truTUBEs) will be called "ASSEMBLY" herein.***



5. Transfer the 96 Shuttle with the truTUBEs to the preheated (56 °C) Dry Block Heater 96 Shuttle equipped with Heat Block Manual 96 Shuttle.
6. Incubate for 5 minutes at 56 °C.

> **NOTE:** All temperatures refer to the sample temperature within the vessel. The Dry Block Heater may need to be adjusted with an offset to achieve the correct temperature. Please see ***Section 2*** for more details.

7. Remove the 96 Shuttle with the truTUBEs and place back into the 16 Home.
8. Place the ASSEMBLY into a benchtop centrifuge equipped with a swinging bucket plate rotor. Counterbalance accordingly.
9. Centrifuge for 10 minutes at 4000 x g.
10. Remove the ASSEMBLY from the centrifuge.
11. Open the truTUBEs with the Decapper truTUBE Screw-Cap and carefully remove the clear Deparaffinization Solution. It is recommended to use a single pipettor for this step. Approximately 470 µL should be removed leaving the blue Rehydration Buffer and up to 60 µL of the Deparaffinization Solution behind. Discard the supernatant.

> **NOTE:** Two phases will be visible after centrifugation. The aqueous phase at the bottom of the tube contains the tissue and a blue dye to make it visible. The Deparaffinization Solution will be on top. When removing the Deparaffinization Solution ensure that the aqueous phase at the bottom of the tube and the interphase are not disturbed.

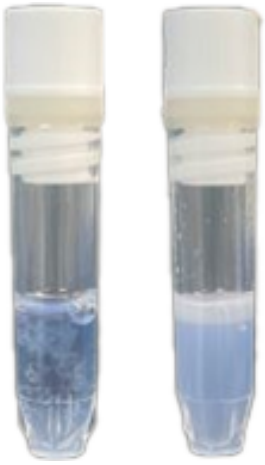
12. ***Optional***: Scan each truTUBE with a barcode reader.

5 - Tissue Homogenization

1. Prepare the Tissue Lysis Buffer/Proteinase K Solution Mix by following instructions in the Table below. Mix by inverting 10 times or vortexing for 3 seconds. The Tissue Lysis Buffer/PK Solution Mix should be stored at ambient temperature and used within 2 hours of preparation.

Reagent	Volume for One Sample*	Volume for N Samples*
Tissue Lysis Buffer	248 µL	248 µL x N
Proteinase K	27.5 µL	27.5 µL x N

*Calculation includes 10% excess in final volume.

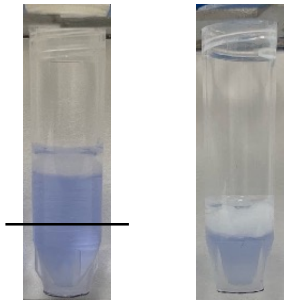


Before AFA After AFA

2. Add 250 µL of the Tissue Lysis Buffer/Proteinase K Solution Mix into each tube. Close the truTUBEs using the Decapper truTUBE Screw-Cap.
3. Use the "Load" position on the instrument. Place the Shuttle Weight on top of the ASSEMBLY to hold the truTUBEs in place. Move the 96 Shuttle with the Shuttle Weight out of the 16 Home and into the Focused-ultrasonicator on top of the PSU Rack R&L-Series 96 Shuttle.
4. Process the samples using the "Acoustic Tissue Homogenization" program.

6 - RNA Fraction Isolation

1. Remove the 96 Shuttle with the Shuttle Weight from the Focused-ultrasonicator and move it to the preheated (56 °C) Dry Block Heater 96 Shuttle equipped with Heat Block Manual 96 Shuttle. Remove the Shuttle Weight.
2. Incubate for 60 minutes at 56 °C.
3. Transfer the 96 Shuttle with the truTUBEs into the 16 Home.
4. Transfer the ASSEMBLY to the benchtop centrifuge equipped with a swinging bucket plate rotor. Counterbalance accordingly.
5. Centrifuge 15 minutes at 4000 x g.
6. Remove the ASSEMBLY from the centrifuge. Do not shake or bump the samples to avoid dislodging the tissue pellet from the tube bottom.
7. This step is critical for a successful experimental outcome. Open the truTUBEs using the Decapper truTUBE Screw-Cap and carefully aspirate 200 µL of the RNA-containing supernatant and transfer it to a 1.5 mL microcentrifuge tube labeled accordingly. The DNA-containing tissue pellet will be located at the bottom of the tube. When removing the supernatant, ensure that the tissue pellet at the bottom of the tube is not disturbed. While aspirating the supernatant, position the pipet tip approximately 7 mm above the bottom of the tube. The black line in the photo shows approximately where to position the pipet tip. If necessary, the sample can be re-centrifuged.



Before Removal After Removal

8. Cap the truTUBEs with the DNA containing pellet using the Decapper and set ASSEMBLY aside for DNA purification. The sample is stable at room temperature for at least 2 hours, or at 2–8 °C for up to 24 hours.
9. Transfer the closed microcentrifuge tubes with the RNA containing supernatant to the preheated (80 °C) Dry Block Heater 16 Microcentrifuge Tube equipped with the Heat Block Manual Microcentrifuge Tube.

> **NOTE:** All temperatures refer to the sample temperature within the vessel. The Dry Block Heater may need to be adjusted with an offset to achieve the correct temperature. Please see ***Section 2*** for more details.

10. Incubate for 30 minutes at 80 °C.
11. Remove the microcentrifuge tubes from the heat block, transfer them to a benchtop centrifuge and centrifuge for 5 seconds to spin down condensation.
12. Proceed to the RNA Purification Section of the protocol.

7 - RNA Purification

For KingFisher semi-automated magnetic bead purification of RNA, please contact applicationsupport@covaris.com

1. Thaw the 10x DNase buffer.
2. Prepare TURBO DNase solution mix according to the table below and pipette mix up and down to mix (10x). Set aside on ice.

Reagent	Volume for One Sample*	Volume for N Samples*
Nuclease Free Water	93.5 µL	93.5 x N µL
10x TURBO DNase Buffer	11 µL	11 x N µL
TURBO DNase	5.5 µL	5.5 x N µL

*Calculation includes 10% excess in final volume.

3. Vortex the Magnetic Bead suspension for 10 seconds.
4. Prepare Binding Mix for RNA according to the table below.

Reagent	Volume for One Sample*	Volume for N Samples*
100% Isopropanol	255 µL	255 x N µL
Magnetic Bead Suspension	8.8 µL	8.8 x N µL
Buffer BB2	110 µL	110 x N µL

*Calculation includes 10% excess in final volume.

NOTE: RNA Yields and DV_{200} Scores: For downstream NGS applications, a lower concentration of Isopropanol may be used to achieve higher DV_{200} scores. See ***Appendix A*** for more details.

5. Vortex the Binding Mix for RNA for 10 seconds immediately before adding it to the samples. Add 340 µL of the Binding Mix to each microcentrifuge tube, cap the tubes and mix thoroughly by vortexing for 15 seconds.
6. Incubate at room temperature for 5 minutes.
7. Place the microcentrifuge tubes onto the Magnetic Stand and wait for at least 3 minutes until the beads have settled on the magnet.
8. Carefully open the tubes while on the magnet; remove and discard the supernatant. Avoid disturbing the bead pellet.
9. Add 800 µL diluted Buffer WB5 to the magnetic bead pellet and resuspend the beads by vortexing.
10. Place the microcentrifuge tubes onto the Magnetic Stand and wait for at least 3 minutes until the beads have settled on the magnet.
11. Open the tubes while on the magnet and remove and discard the supernatant. Remove as much supernatant as possible without disturbing the bead pellet.
12. Remove the tubes from the magnet and add 100 µL DNase solution mix.
13. Resuspend beads by pipetting up and down (10x) or until beads are completely resuspended.
14. Close the tubes and incubate for 30 minutes at room temperature.
15. Open the tubes and add 50 µL Buffer BB2 to each sample.
16. Add 100 µL 100% Isopropanol to each sample, close the tubes and vortex for 10 seconds.

17. Incubate at room temperature for 5 minutes.
18. Place the microcentrifuge tubes onto the Magnetic Stand and wait for at least 3 minutes until the beads have settled on the magnet.
19. Open the tubes and remove and discard the supernatant. Avoid disturbing the bead pellet.
20. Remove the tubes from the magnet and add 800 µL diluted Buffer WB5 to the magnetic bead pellet.
21. Close the tubes and resuspend the beads by vortexing.
22. Place the microcentrifuge tubes onto the Magnetic Stand and wait for at least 2 minutes until the beads have settled on the magnet.
23. Open the tubes and remove and discard the supernatant. Avoid disturbing the bead pellet.
24. Remove the tubes from the magnet and add 800 µL 80% Ethanol to each sample.
25. Close the tubes and resuspend the beads by vortexing.
26. Place the microcentrifuge tubes onto the Magnetic Stand and wait for at least 2 minutes until the beads have settled on the magnet.
27. Open the tubes and remove and discard the supernatant. Avoid disturbing the bead pellet.
28. Remove the tubes from the magnet and add 500 µL 80% Ethanol to each sample.
29. Close the tubes and resuspend the beads by vortexing.
30. Place the microcentrifuge tubes onto the Magnetic Stand and wait for at least 2 minutes until the beads have settled on the magnet.
31. Open the tubes and remove as much of the 80% Ethanol as possible without disturbing the bead pellet.
32. Leave the tubes on the magnet with the lids open and dry the beads for 5 minutes.
33. Remove tubes from the magnet.
34. Add 50 µL RNA Elution Buffer to each sample and resuspend beads by pipetting up and down 10 times (beads must be completely resuspended).
35. Close the tubes and incubate at room temperature for 3 minutes.
36. Place the microcentrifuge tubes onto the Magnetic Stand and wait for at least 2 minutes until the beads have settled on the magnet.
37. Open the tubes and transfer the supernatant that contains the eluted RNA to a LoBind tube or a PCR tube and store appropriately to ensure RNA stability.

8 - DNA Fraction Isolation

1. Prepare the Tissue Lysis Buffer/Proteinase K Solution Mix for DNA according to the Table below. Mix by inverting 10 times or vortexing for 3 seconds. The Tissue Lysis Buffer/Proteinase K Solution Mix should be stored at ambient temperature and used within 2 hours after preparation.

Reagent	Volume for One Sample*	Volume for N Samples*
Tissue Lysis Buffer	198 µL	198 x N µL
Proteinase K Solution	44 µL	44 x N µL

*Calculation includes 10% excess in final volume.

2. Retrieve the ASSEMBLY containing the truTUBEs with the DNA containing tissue pellets from storage.
3. Open the truTUBEs using the Decapper truTUBE Screw-Cap and add 220 µL of the Tissue Lysis Buffer/Proteinase K Solution Mix for DNA. Close the truTUBEs using the Decapper truTUBE Screw-Cap.
4. Place the Shuttle Weight on top of the ASSEMBLY to hold the truTUBEs in place.
5. Use the “Load” position on the instrument. Move the 96 Shuttle with the Shuttle Weight into the Focused-ultrasonicator on top of the PSU Rack R&L-Series 96 Shuttle.
6. Process the samples using the “Acoustic Pellet Resuspension” program.
7. Remove the 96 Shuttle with the Shuttle Weight from the Focused-ultrasonicator and transfer to the preheated (56 °C) Dry Block Heater 96 Shuttle equipped with Heat Block Manual 96 Shuttle. Remove the Shuttle Weight.
8. Incubate for 60 minutes at 56 °C.
9. Transfer the 96 Shuttle into the 16 Home and uncap the truTUBEs using the Decapper truTUBE Screw-Cap.
10. Mix the sample by pipetting up and down 3 times and transfer it completely into a 1.5 mL microcentrifuge tube, labeled accordingly.
11. Move the microcentrifuge tubes into a preheated (80 °C) Dry Block Heater 16 Micocentrifuge Tube equipped with Heat Block Manual Microcentrifuge Tube.
12. Incubate for 60 minutes at 80 °C.
13. Remove the microcentrifuge tubes from the heat block, transfer them to a benchtop centrifuge and centrifuge for 5 seconds to spin down condensation.
14. ***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 4 µL of RNase A (10 mg/mL) solution and incubate for 5 minutes at room temperature.
15. Proceed to the DNA purification section.
16. ***IMPORTANT***: Set the temperature of a Dry Block Heater 16 Microcentrifuge Tube equipped with Heat Block Manual Microcentrifuge Tube to 56 °C. This will be needed for DNA elution (***Section 9 - DNA Purification, Step 25***).

9 - DNA Purification

For KingFisher semi-automated magnetic bead purification of DNA, please contact applicationsupport@covaris.com

1. Vortex the Magnetic Bead Suspension for 10 seconds.
2. Prepare the Binding Mix for DNA according to the table below.

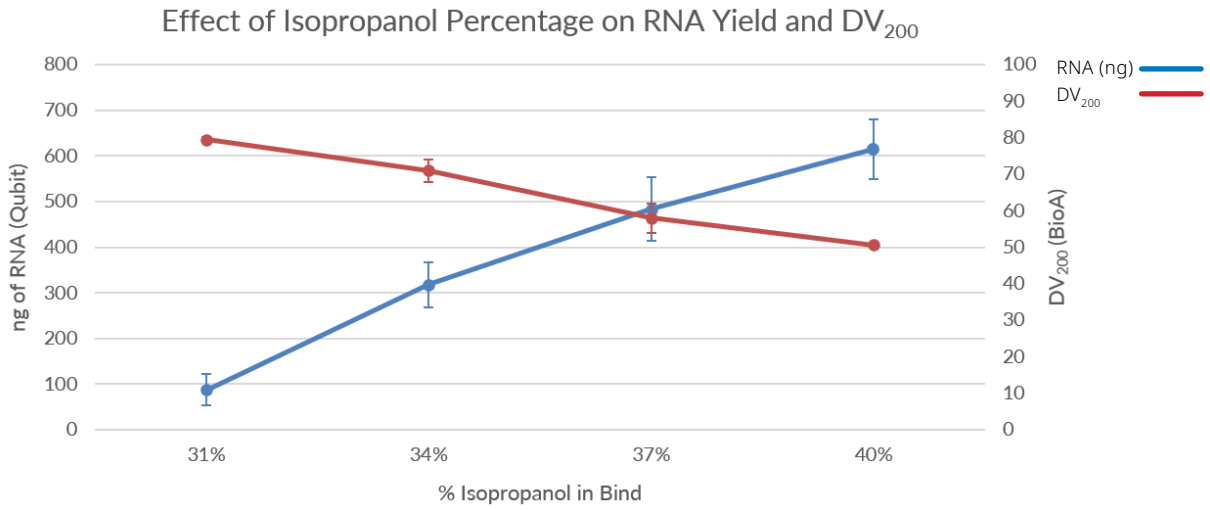
Reagent	Volume for One Sample*	Volume for N Samples*
100% Isopropanol	330 µL	330 x N µL
Magnetic Bead Suspension	8.8 µL	8.8 x N µL
Buffer BB2	137.5 µL	137.5 x N µL

*Calculation includes 10% excess in final volume.

3. Vortex the Binding Mix for DNA for 10 seconds immediately before adding it to the samples. Open the tubes and add 433 µL of the Binding Mix to each microcentrifuge tube.
4. Cap the tubes and mix thoroughly by vortexing for 15 seconds.
5. Incubate at room temperature for 5 minutes.
6. Place the microcentrifuge tubes onto the Magnetic Stand and wait for at least 3 minutes until the beads have settled on the magnet.
7. Open the tubes and carefully remove and discard the supernatant. Avoid disturbing the bead pellet.
8. Remove the tubes from the magnet and add 800 µL diluted Buffer WB5 to the magnetic bead pellet.
9. Close the tubes and resuspend the beads by vortexing.
10. Place the microcentrifuge tubes onto the Magnetic Stand and wait for at least 2 minutes until the beads have settled on the magnet.
11. Open the tubes and carefully remove and discard the supernatant. Avoid disturbing the bead pellet.
12. Repeat steps 8–11.
13. Remove the tubes from the magnet and add 800 µL 80% Ethanol to each sample.
14. Cap the tubes and resuspend the beads by vortexing.
15. Place the microcentrifuge tubes onto the Magnetic Stand and wait for at least 3 minutes until the beads have settled on the magnet.
16. Open the tubes and remove and discard the supernatant. Avoid disturbing the bead pellet.
17. Remove the tubes from the magnet and add 500 µL 80% Ethanol to each sample.
18. Cap the tubes and resuspend the beads by vortexing.
19. Place the microcentrifuge tubes onto the Magnetic Stand and wait for at least 2 minutes until the beads have settled on the magnet.
20. Open the tubes and remove as much of the 80% Ethanol as possible without disturbing the bead pellet.
21. Leave the tubes on the magnet with the lids open and dry the beads for 5 minutes.
22. Remove tubes from the magnet and add 50 µL Buffer BE to each sample.
23. Resuspend beads by pipetting up and down 10 times (beads must be completely resuspended).
24. Move the microcentrifuge tubes into a preheated (56 °C) Dry Block Heater 16 Microcentrifuge Tube equipped with Heat Block Manual Microcentrifuge Tube.
25. Incubate at 56 °C for 5 minutes.
26. Place the samples on the magnet stand and let sit for 2 minutes.
27. Open the tubes and transfer supernatant that contains the eluted DNA to a LoBind tube or a PCR tube.

Appendix A: Isopropanol Concentration and DV_{200} Scores of the Recovered RNA

The isopropanol concentration used in ***Section 7*** (RNA Purification) will impact RNA yield and size distribution (as expressed by DV_{200} score [3]). If high DV_{200} scores are desirable, use a lower concentration of isopropanol in the bind mix. However, if maximum RNA yield is desired at the expense of the DV_{200} score (increase of <200nts RNA fraction), use a higher concentration of isopropanol. Higher Isopropanol concentrations will bind more fragments with lower molecular weight to the beads. An example is shown in the figure below: RNA extraction from an FFPE liver scroll with mostly small RNA fragments. Increasing the Isopropanol concentrations in the bind mix results in a higher yield and a lower DV_{200} score.



The table below shows different isopropanol volumes and final isopropanol concentrations. The default isopropanol concentration used for RNA purification is 43%. The amount of isopropanol added to the Binding Mix for RNA Table in ***Section 7*** can be decreased if a higher DV_{200} is desired.

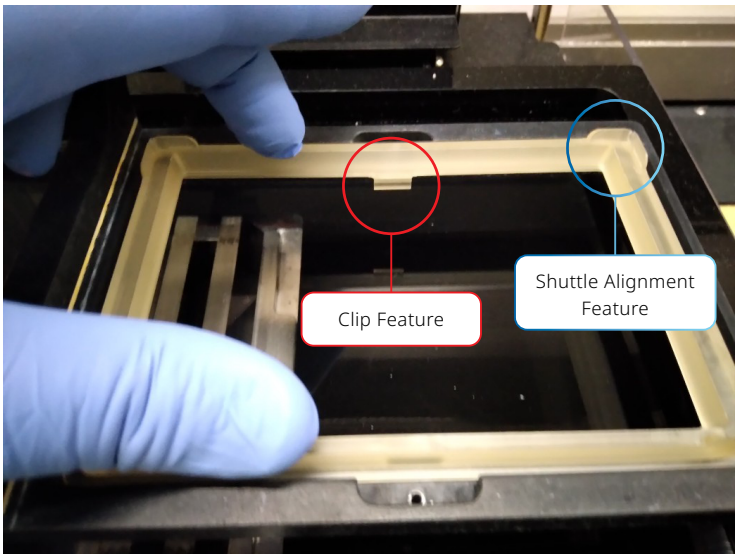
Volume for One Sample

Isopropanol in Bind (%)	33	37	40	43
100% Isopropanol*	171 µL	198 µL	226 µL	255 µL
Buffer BB2*	110 µL	110 µL	110 µL	110 µL
Magnetic Bead Suspension*	8.8 µL	8.8 µL	8.8 µL	8.8 µL
µL of mix to add to each sample	**263 µL**	**288 µL**	**313 µL**	**340 µL**

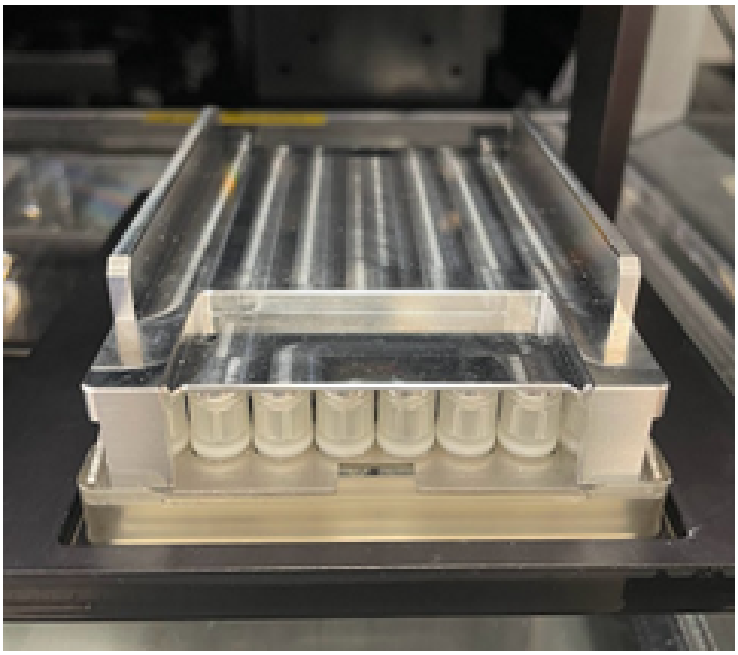
*Calculation includes 10% excess in final volume.

Appendix B: PSU Rack R&L-Series 96 Shuttle (PN 500732) and Shuttle Weight (PN 500795)

- The PSU Rack R&L- Series 96 Shuttle (PN 500732) has Clip Features that allow it to snap into the Covaris R230 and L-Series Focused-ultrasonicators.
  - These features are located on the bottom of the Rack at the midpoint of the longer sides of the Rack.
  - The clip features should face down into the water bath and the Rack should be gently pushed into the sample arm of the instrument until the clips engage with the sample arm holding the Rack in place.
- The PSU Rack R&L- Series 96 Shuttle (PN 500732) has Shuttle Alignment Features to guide the 96 Shuttle into position for AFA when placed onto the Rack.
  - These features are located on the top of the Rack in all four corners.
  - When inserting the Rack into the Sample arm these should face up away from the water bath.



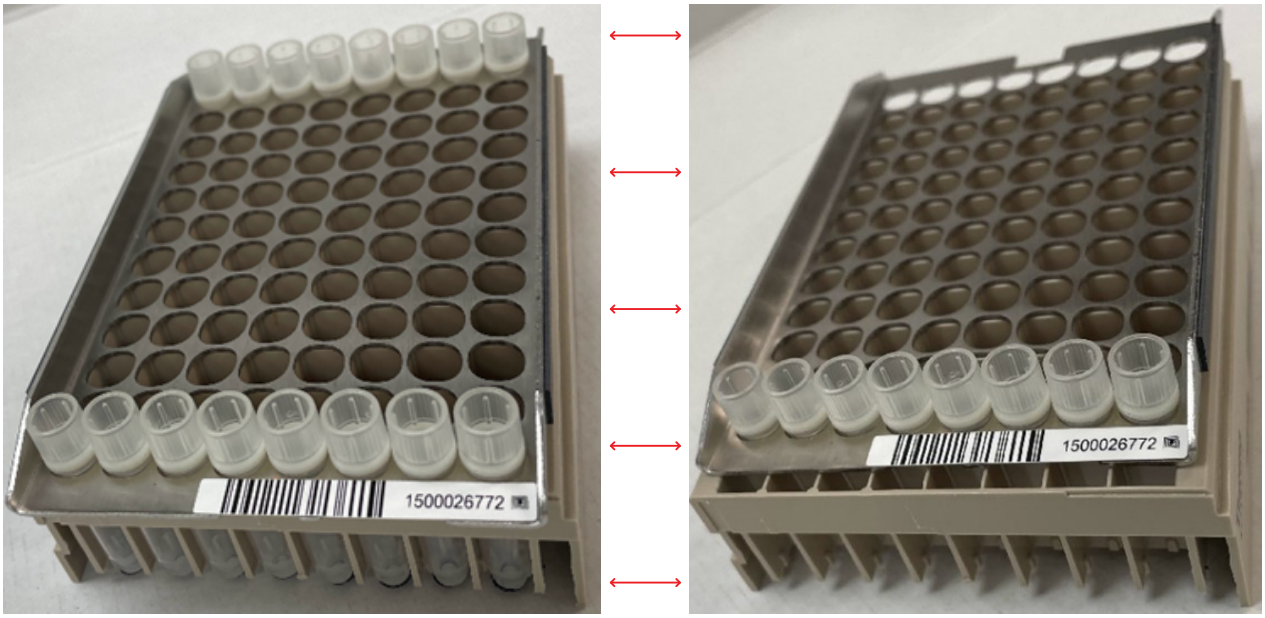
The Shuttle Weight (PN 500795) should be used along with the PSU Rack when processing samples. A photo of the complete set-up is shown below in an L-series Focused-ultrasonicator.



Appendix C: Decapping and Capping of truTUBEs in the 96 Shuttle

The 96 Shuttle is designed for on-deck automation as the truTUBE Screw-Cap tubes sit loose in the 96 Shuttle. The provided Decapper truTUBE Screw-Cap tool can be used as described below.

- Lift up the 96 Shuttle and shift the tubes over 1 column:
  - Move from column 1 to 2 for decapping or capping column 1, or move from column 12 to 11 for decapping or capping column 12.
- After decapping or capping the Screw-Caps, the 96 Shuttle can be shifted back to the original location before continuing the protocol.



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

- To determine RNA and DNA yields, a fluorometric assay such as Qubit™ (Life Technologies) should be used.
- In addition, spectrophotometric analysis determining the A260/280 and A260/230 ratios will determine if protein or peptide / salt contamination is present in the sample.
- qPCR or RT-qPCR can be used to assess the amplifiability of isolated DNA and RNA as well as the presence of inhibitors. Note that DNA from FFPE tissue itself can act as an inhibitor at high input concentrations due to the extensive damage (e.g., nicks and/or depurination). Therefore, a dilution series over at least 5 orders of magnitude starting with undiluted material of the extracted DNA should always be done when assessing quality by qPCR. An example is shown in Dietrich et al. Figure 1 [4].

Additional Notes

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

References

1. Carrick et al. (2015) Robustness of Next Generation Sequencing on Older Formalin-Fixed Paraffin-Embedded Tissue. PLoS ONE 10(7): e0127353.
2. Kresse et al. (2018). Evaluation of commercial DNA and RNA extraction methods for high-throughput sequencing of FFPE samples. PLoS ONE 13(5): e0197456.
3. Landolt et al. (2016) RNA extraction for RNA sequencing of archival renal tissues. Scand J Clin Lab Invest 76(5):426-434.
4. Dietrich et al. (2013) Improved PCR Performance Using Template DNA from Formalin-Fixed and Paraffin-Embedded Tis sues 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, during the hours of 8:30 a.m. to 5:00 p.m. (EST), Monday through Friday
  - EU: +44 (0)845 872 0100, during the hours of 9:00 a.m. to 5:00 p.m. (GMT), Monday through Friday
- **E-mail:**
  - Service and Instrumentation: techsupport@covaris.com
  - Solutions: applicationsupport@covaris.com
  - US Customer Service: customerservice@covaris.com
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