PROTOCOL

Covaris

truXTRAC™ FFPE total NA Kit - Column Purification (25)

Adaptive Focused Acoustics™ (AFA) -based sequential RNA and DNA extraction from FFPE tissues using column-based purification

Product PN 520220

Contents

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). RNA isolation is followed by DNA isolation 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, allowing simultaneous tissue rehydration. Compared to traditional passive, chemical-based methods of paraffin removal, this mechanical process is not as limited by the thickness of FFPE tissue sections. Thus, the use of AFA enables input of thicker FFPE sections, which results in increased yields of nucleic acids, whilst minimizing the degradation of nucleic acids exposed at the FFPE section surface. The truXTRAC process results in high yields of high-quality total NA well suited for analytical methods such as next-generation sequencing (NGS) or qPCR/qRT-PCR.

This protocol is optimized for sections up to 25 µm in thickness.

Important Notes on FFPE Samples:

The yield of DNA and RNA from FFPE tissue blocks can be highly variable. Factors such as fixation time, size and thickness of the sections, 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.

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. Incomplete de-crosslinking may cause the isolated total NA to perform less well in downstream applications such as PCR, qPCR, qRT-PCR or NGS. In addition, the size of NA fragments isolated from FFPE samples is generally smaller than that of total NA isolated from fresh or frozen tissues [1]. This is particularly evident in older FFPE sample blocks or sample blocks stored at elevated temperatures.

Note for first time users:

Given the highly variable yield of total NA from FFPE tissue blocks, we recommend using FFPE blocks that have been well characterized for yield and quality for initial testing of the truXTRAC FFPE total NA kit. Ideally, samples should be extracted immediately after sectioning.

If you require any help with this product please 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
010373	A	07/17	Initial release of truXTRAC FFPE total NA - Column Purification Kit

KIT CONTENTS

Tissue Lysis Buffer	6 ml
Proteinase K (PK) Solution	0.96 ml
B1 Buffer	15 ml
RNA Wash Buffer	10 ml
RNA elution buffer	3 ml
BW Buffer	15 ml
B5 Buffer	7 ml
DNA Elution Buffer (Buffer BE)	7.5 ml
RNA purification columns	25
DNA purification columns	25
Collection Tubes	50
RNA Elution Tubes	25
microTUBE-130 AFA Fiber Screw-Cap FFPE	25

SDS INFORMATION IS AVAILABLE AT http://covaris.com/resources/safety-data-sheets/

STORAGE

Store PK solution at 2-8°C.

Store all other Kit components at room temperature.

SUPPLIED BY USERS

Covaris Instruments and Parts

Required parts						
Focused-ultrasonicator	**LE220**	**E220 & E210**	**E220 evolution**	**S-Series**	**M220**	**ME220**
Rack/ Holder	Rack-XT 24 Place microTUBE Screw-Cap PN500388	Rack 24 Place microTUBE Screw-Cap PN500308	Rack E220e 4 Place microTUBE Screw Cap PN500432	Holder microTUBE Screw-Cap PN500339	Holder XTU PN500414 & Insert XTU PN500489 **(*)**	Rack 4-place microTUBE Screw-Cap PN500522
Intensifier	NA	PN500141	PN500141	NA	NA	Waveguide 4 Place PN500534
Accessories	Centrifuge and Heat Block microTUBE Adapter (PN500406)					
Optional parts						
Accessories	FFPE tissuePICK (PN520163) tissuePICK Forceps (5) (PN 520164) FFPE sectionPICK (PN520149) FFPE sectionWARMER (PN500403)					

(*) Holder XT PN500358, although discontinued, can be used. This holder does not require an insert.

Other supplies (not provided in this kit):

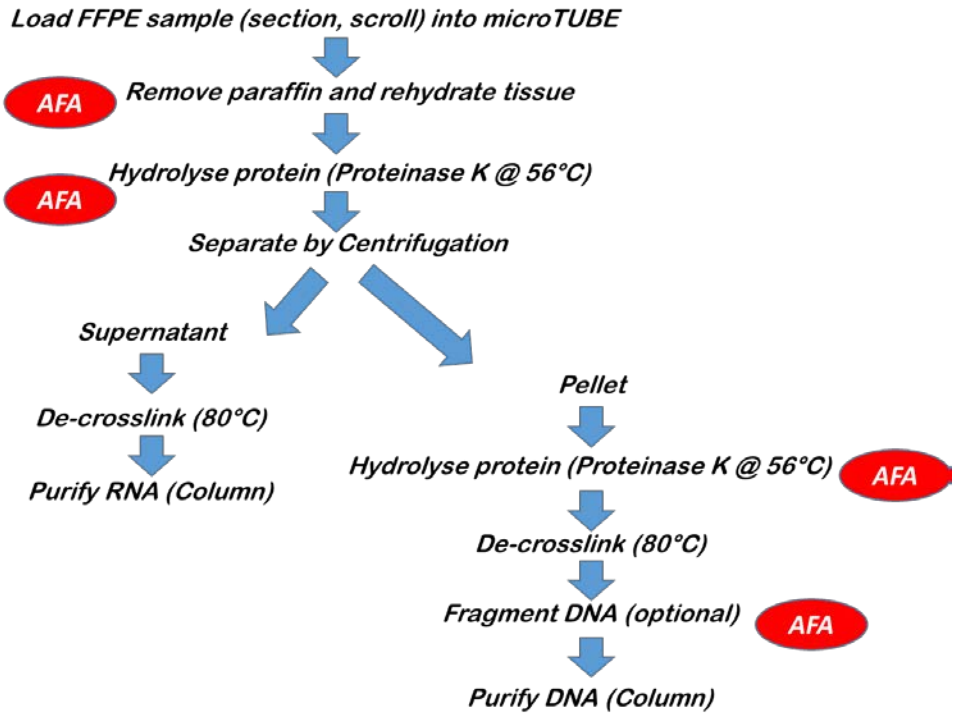
- Microcentrifuge with 16,000 x g capability
- 1.5 ml nonstick nuclease free microfuge tubes (e.g. Life Technologies; PN AM12450)
- Water bath, oven or dry block heater with a heated cover, capable of heating to 80°C (e.g. Eppendorf ThermoMixer C)
  Covaris recommends two heating devices, preset to 56°C and 80°C, respectively
- Optional RNase A (DNase free) at 10 mg/ml (e.g. Thermo Scientific; PN EN0531)
- Optional TURBO DNA-*free* kit™ (Thermo Scientific; PN AM1907)
- Isopropanol, ultrapure or MB grade (e.g. AmericanBio; PN AB07015)
- Ethanol (>96%), MB Grade (e.g. Thermo Scientific; PN BP2818-100)
- Nuclease-free water (e.g. Ambion; PN AM9930)

PROCEDURE WORKFLOW OVERVIEW

The diagram below outlines the truXTRAC FFPE total NA extraction workflow. Using AFA, FFPE tissue sections are emulsified in Tissue Lysis Buffer followed by addition of Proteinase K, AFA induced mixing, and incubation at 56°C for 15 minutes. This results in RNA extraction, with minimal release of genomic DNA.

After brief centrifugation, RNA-containing supernatant is separated from the DNA containing solids, de-crosslinked, and column-purified.

Subsequently, DNA can be extracted using AFA from the remaining FFPE tissue and column-purified.



1 – PREPARATION

FFPE Tissue Sample

1. Sample Input requirements

The truXTRAC process is highly efficient at removing paraffin, even from relatively thick FFPE sections, while simultaneously rehydrating the tissue. Use of thicker sections is often desirable, not only for increased yields, but also due to the quick degradation of DNA/RNA found on exposed surfaces of a section. Covaris recommends using sections between 15 and 25 µm thick.

NOTE: Excess paraffin will adversely affect the yield and quality of DNA and RNA extracted from FFPE specimens. We strongly advise trimming off any excess paraffin before sectioning a FFPE tissue block, or after the section has been cut from the FFPE block. A ratio of 80% tissue to 20% paraffin, or higher, is ideal.

	FFPE Sections Mounted on slide		FFPE Sections scrolls	
Size (thickness or diameter)	4 to 10 µm	7 to 10 µm	7 to 15 µm	16 to 25 µm
Size (length)	NA		<10 mm (Note A)	
Collection tool	tissuePICK (Note B)	sectionPICK (Note B)	NA	
Maximum number of samples per microTUBE	2x tissuePICK (200 mm^2 tissue for a 5 µm section)	2x sectionPICK	2*	1*

* Numbers represent trimmed sections only

NOTES

A. If the FFPE sample is longer than 10 mm, cut the section in half or fold before loading.

B. For optimal tissuePICK and sectionPICK performances, the tissue section should be mounted on uncoated slides. tissuePICK and sectionPICK should always be used in conjunction with a sectionWARMER.

WARNING: The total mass of FFPE sample processed per extraction should be between 2 to 5 mg. Lower amounts may result in insufficient yield and higher amounts may cause spin columns to become partially or fully clogged.

2. Tissue Fixation Requirements

The yield and quality of DNA/RNA extracted from FFPE tissue blocks is highly dependent on tissue collection and paraffin embedding procedures. For good yields of high quality DNA/RNA:

- Do not exceed a fixation time of 24 hours
- Use 4% neutral buffered formalin solution
- Fix tissue sample as quickly as possible after collection

Buffer Preparations

1. **RNA Wash Buffer:** Add 40 ml of 100% ethanol to 10 ml RNA Wash Buffer and mark the bottle label accordingly. After preparation, the RNA Wash Buffer can be stored for up to one year at room temperature. Minimize the number of times the bottle is opened to avoid evaporation of ethanol.
2. **B5 Buffer**: Add 28 ml of 100% ethanol to 7 ml B5 Buffer and mark the bottle label accordingly. After preparation, the B5 Buffer can be stored for up to one year at room temperature. Minimize the number of times the bottle is opened to avoid evaporation of ethanol.
3. **65% Isopropanol:** Nuclease-free water and ultrapure or MB Grade isopropanol should be used (e.g. Add 35 ml nuclease-free water to 65 ml isopropanol).
4. **B1 Buffer and Tissue Lysis Buffer**: Check these buffers visually, as a white precipitate may form during storage. If white precipitate is visible, incubate the buffer bottles at 50 – 60 °C for 5 to 10 minutes before use to dissolve any precipitate.

Focused-ultrasonicator

NOTE: For detailed instructions on how to prepare your particular instrument, please refer to your instrument's User Manual.

- For S, E or LE-Series Focused-ultrasonicators, set up the instrument as shown in the table below. Wait for the water to reach temperature and to be sufficiently degassed.

Focused-ultrasonicator setup

Instrument	Water level*	Chiller temp	Intensifier	Plate definition**	Holder or Rack
S-Series	**15**	**18°C**	**NA**	NA	PN500339
E220 & E210	**10**	**18°C**	**Yes**	500308 Rack 24 Place microTUBE Screw-Cap	PN500308
E220 evolution	**10**	**18°C**	**Yes**	500432 Rack E220e 4 Place microTUBE Screw Cap	PN500432
L-Series	**10**	**18°C**	**NA**	500388 Rack-XT 24 Place microTUBE Screw-Cap	PN500388

* Use RUN side of FILL/RUN water level label when transducer is submerged.

**If you do not see a plate definition on your system, please contact Covaris Technical Support at TechSupport@covarisinc.com.

- For M220 Focused-ultrasonicators, position Holder XTU PN500414 and Insert PN500489 (or the discontinued Holder XT PN500358 without insert) into place and fill the water bath until the water reaches the top of the holder. Allow system to reach 20°C.
- For ME220 Focused-ultrasonicators, position the ME220 Waveguide 4 Place PN500534 into place in the water bath. Allow system to reach 20°C. Load samples into Rack 4 Place PN500522 and place into the rack holder.

Heating Block, Water Baths

Preheat dry block heaters or water baths to 56°C (or T set1 - see Appendix C) and 80°C (or T set2 - see Appendix C).

When using a dry block heater, microTUBEs must to be placed in Centrifuge and Heat Block microTUBE Adapters (PN500406).

It is important to confirm that the desired temperatures are actually reached. See Appendix C for instructions on how to calibrate your heating device.

2 – Paraffin Emulsification, Tissue Rehydration & Lysis

1. Open microTUBE Screw-Cap, add 110 µl Tissue Lysis Buffer, and load FFPE tissues.

> **NOTE**: If the FFPE tissue samples are loose or broken, the tissue may also be added to the microTUBE prior to addition of the Tissue Lysis Buffer to facilitate easier loading.

2. Close microTUBE tightly with the Screw-Cap.
3. Process the sample in the Focused-ultrasonicator, using the settings provided in Table 1 below, to dissociate the paraffin while simultaneously rehydrating the tissue.

Table 1. AFA Settings for Paraffin Emulsification and Tissue Rehydration

System	Duty Factor	Peak Incident Power	Cycles per burst	Treatment Time	Temperature (Instrument)
S220 or E220	10%	175 Watts	200	300 sec	20 °C
S2 or E210	10%	5 (Intensity)	200	300 sec	20 °C
M220	20%	75 Watts	200	300 sec	20 °C
ME220	25%	75 Watts	1000	390 sec	20 °C
LE220(1)	30%	450 Watts	200	300 sec	20 °C

(1) As the LE220 Focused-ultrasonicator can process multiple samples simultaneously, the power is distributed across the microTUBEs. Power received by individual microTUBEs stays within the 200 Watt limit.

> **NOTE**: During the AFA-based emulsification and rehydration it is normal for the solution to turn milky white. Please see the example in Appendix A.

4. Open the microTUBE, add 10 µl of PK solution to the sample, and close the microTUBE Screw-Cap again tightly.
5. Process the sample in the Focused-ultrasonicator, using the settings provided in Table 2 below, to homogenize PK with the sample.

Table 2. AFA settings for PK/Sample homogenization

System	Duty Factor	Peak Incident Power	Cycles per burst	Treatment Time	Temperature (Instrument)
S220 or E220	10%	175 Watts	200	10 sec	20 °C
S2 or E210	10%	5 (Intensity)	200	10 sec	20 °C
M220	20%	75 Watts	200	10 sec	20 °C
ME220	25%	75 Watts	1000	10 sec	20 °C
LE220	30%	450 Watts	200	10 sec	20 °C

6. PK digestion:
    a. Insert the required number of Centrifuge and Heat Block microTUBE Adapters into a heat block (e.g. Eppendorf ThermoMixer C).
    b. Load the microTUBE into the adapter once the heat block has reached its set point
    c. Incubate for 15 minutes at 56°C, remove from the heat block, and cool to room temperature.

7. Spin in a microcentrifuge at 5,000 x g for 15 minutes

NOTE: The Centrifuge and Heat Block microTUBE Adapters PN500406 must be used

8. Open the microTUBE and carefully transfer 100 µl supernatant, without disturbing the pellet, to a clean 1.5 ml RNase-free microcentrifuge tube. Proceed to RNA purification immediately.

9. Keep the pellet for subsequent DNA purification as described in Section 4. The DNA-containing pellet can be stored on ice or at 2–8°C for up to 1 day. For longer periods, store at –15 to –30°C.

3 – RNA PURIFICATION

1. Incubate the 1.5 ml microcentrifuge tube containing the RNA within the supernatant at 80°C in a covered heat block or water bath for 15 minutes, then cool to room temperature.
2. While the samples are incubating, prepare RNA Purification Columns by inserting them into the provided Collection Tubes.
3. After incubation, add 175 µl B1 Buffer to the heat-treated RNA supernatant and mix by pipetting for 3 seconds.
4. Add 250 µl 65% isopropanol to the samples and mix by pipetting for 3 seconds.

NOTE: For downstream NGS applications a lower concentration of isopropanol may be used to achieve higher DV200 scores [2]. Conversely, for maximum RNA yield with the sacrifice of DV200 scores, use more concentrated isopropanol. See Appendix E for more details.

5. Transfer the entire sample to the RNA Purification Column.

NOTE: Small amounts of residual wax will not interfere with the column purification.

6. Centrifuge the Column/Collection Tube assembly at 11,000 x g for 30 seconds.
7. Discard the flow-through and place the Column back into the Collection Tube.

8. **1st wash**:
    a. Add 700 µl of prepared RNA Wash Buffer to the RNA Purification Column.
    b. Centrifuge the Column/Collection Tube assembly at 11,000 x g for 30 seconds.
    c. Discard the flow-through and place the Column back into the Collection Tube.

Optional DNA removal step:
truXTRAC FFPE total NA Kit protocol isolates total RNA that contains small amounts of genomic DNA. If DNA-free RNA must be isolated an optional DNase treatment can be performed. Two different protocols are provided in Appendix D.
If choosing the on-column DNase digestion, it must be performed after step 8.
If choosing the in-solution DNase treatment it must be performed after step 11.

9. **2nd wash**:
    a. Add 250 µl of prepared RNA Wash Buffer to the RNA Purification Column.
    b. Centrifuge the Column/Collection Tube assembly at 16,000 x g for 1 minute.

10. **RNA elution**:
    a. Place the Column into a new RNA Elution Tube (1.5 ml) and add 30 µl (for high concentration) or 50 µl (for high yield) RNA elution buffer to the center of the Column.
    b. Make sure the solution spreads throughout the membrane on the bottom of the Columns. A brief vortex can be used to achieve thorough membrane wetting.
    c. Incubate for 2 minutes at room temperature.
    d. Spin the Column/Collection Tube assembly at 16000 x g for 1 minute.

11. Keep the eluted RNA on ice for further processing. Isolated RNA should be kept at -80^{o}C for long term storage.

4 – DNA PURIFICATION

1. Prepare DNA Lysis Buffer by adding 22 µl of PK solution to 88 µl of Tissue Lysis Buffer in a 1.5 ml microcentrifuge tube. If processing multiple samples at the same time, multiply respective volumes by the number of samples. (Note: In step 1 DNA Lysis Buffer is prepared 10% more due to pipette inaccuracies)
2. Pre-warm required volume of Buffer BE in a 1.5 ml microcentrifuge tube at 70°C. Continue to warm the buffer until you reach step 19.
3. Open the microTUBE that contains the FFPE tissue pellet (the tube labeled with DNA) and add 100 µl of the DNA Lysis Buffer. Re-cap the Screw-Cap microTUBE tightly.
4. Process the sample in the Focused-ultrasonicator, using the settings provided in Table 3 below, to mix DNA Lysis Buffer and FFPE tissue.

Table 3. DNA Lysis Buffer/FFPE tissue homogenization settings.

System	Duty Factor	Peak Incident Power	Cycles per burst	Treatment Time	Temperature (Instrument)
S220 or E220	**10%**	**175 Watts**	**200**	**10 sec**	**20 °C**
S2 or E210	**10%**	**5 (Intensity)**	**200**	**10 sec**	**20 °C**
M220	**20%**	**75 Watts**	**200**	**10 sec**	**20 °C**
ME220	**25%**	**75 Watts**	**1000**	**10 sec**	**20 °C**
LE220	**30%**	**450 Watts**	**200**	**10 sec**	**20 °C**

5. PK digestion (Heat block pre-set to 56°C):
    a. Insert the required number of Centrifuge and Heat Block microTUBE Adapters into a heat block
    b. Load the microTUBE into the adapter once the heat block has reached its set point
    c. Incubate for 60 minutes at 56°C.

6. De-crosslinking (Heat block pre-set to 80°C):
    a. Insert the required number of Centrifuge and Heat Block microTUBE Adapters into a heat block
    b. Load the microTUBE into the adapter once the heat block has reached its set point
    c. Incubate for 60 minutes at 80°C, remove from the heat block and cool to room temperature.

7. Transfer the sample to a clean 1.5 ml microcentrifuge tube.

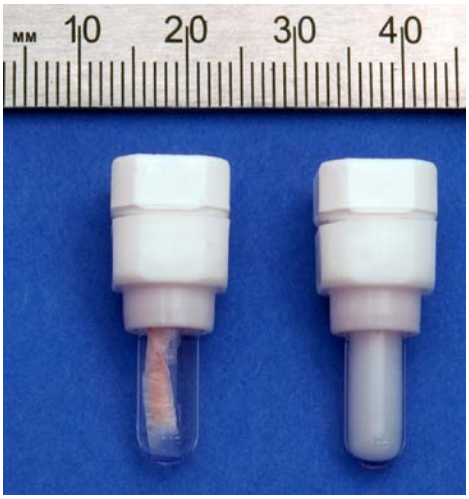
Optional RNA removal step:
At this point the sample can be treated with RNase A to remove residual RNA before continuing with DNA purification.
Add 5µl of RNase A solution and incubate for 5 minutes at room temperature then continue to step 8.

8. Add 140 µl B1 Buffer to the sample and vortex for 3 seconds.
9. Add 160 µl >96% ethanol to the sample and vortex for 3 seconds.
10. Centrifuge at 10,000 x g for 2 minutes at room temperature.
    (After centrifugation, most of the paraffin will have formed a white layer, floating on top of the liquid).
11. Place a DNA Purification Column into a Collection Tube.
12. While holding the sample tube at roughly the same angle as the centrifuge rotor, use a pipette to slowly recover the liquid layer, and transfer to the Purification Column.
13. Spin the Column/Collection Tube assembly at 11,000 x g for 1 minute.
14. Discard the flow-through and place the Column back into the Collection Tube.

15. **1st wash**:
    a. Add 500 µl BW Buffer to the DNA Purification Column.
    b. Spin the assembly at 11,000 x g for 1 minute.
    c. Discard the flow-through and place the Column back into the Collection Tube.
16. **2nd wash**:
    a. Add 600 µl of prepared B5 Buffer to the DNA Purification Column.
    b. Spin the assembly at 11,000 x g for 1 minute.
    c. Discard the flow-through and place the Column back into the Collection Tube.
17. **Dry column**: Spin the assembly at 11,000 x g for 1 minute.
18. **Elute DNA - 1st step**:
    a. Place the Purification Column into a clean 1.5 ml microcentrifuge tube.
    b. Add 50 µl of pre-warmed Buffer BE (70 °C) to the center of the Column.
    c. Leave Column at room temperature for 3 minutes.
    d. Spin the Column/microcentrifuge tube assembly at 11,000 x g for 1 minute.
19. **Elute DNA – 2nd step**:
    a. Add a second 50 µl aliquot of pre-warmed Buffer BE (70 °C) to the center of the Column.
    b. Incubate again at room temperature for 3 minutes.
    c. Spin the Column/microcentrifuge tube assembly at 11,000 x g for 1 minute.

APPENDIX A – EXAMPLE OF AFA-BASED PARAFFIN EMULSIFICATION AND TISSUE REHYDRATION

The picture below represents how the FFPE tissue appears before (left side) and after (right side) AFA processing. Paraffin in the FFPE tissue (10 µm kidney tissue section) was emulsified in a microTUBE Screw-Cap using a Covaris S220 Focused-ultrasonicator.



APPENDIX B – TROUBLESHOOTING GUIDE

Issue	Cause	Solution	Comments / Suggestions
Low yield of RNA and/or DNA	Low tissue to wax ratio in FFPE section.	Trim off any excess paraffin before sectioning a FFPE tissue block. Repeat the procedure using additional sections until desired yield is achieved.	In your initial use of the truXTRAC FFPE total NA kit use FFPE blocks that have been well characterized for yield and quality.
	Insufficient tissue input.	Increase FFPE tissue section thickness or use additional sections up to 5mg total weight.	
	PK solution stored above recommended temperature or expired.	Repeat the procedure using fresh PK solution.	Always store PK solution as recommended.
No RNA	Ethanol not added to RNA Wash Buffer.	Repeat the procedure with fresh samples and ensure ethanol is added to RNA Wash Buffer.	
RNA concentration is too low	Elution volume is too high.	Repeat procedure using a lower elution volume (30 µl minimum volume is required). Alternatively, concentrate samples using ethanol precipitation or other suitable volume reduction methods.	
No DNA	Ethanol not added to B5 Buffer.	Repeat the procedure with fresh samples and ensure ethanol is added to B5 Buffer.	
DNA concentration is too low	Elution volume is too high.	Repeat procedure using a lower elution volume (50 µl minimum volume is required). Alternatively, concentrate samples using ethanol precipitation or other suitable volume reduction methods.	
DNA does not perform well in downstream applications such as qPCR	DNA in FFPE sample blocks is severely cross-linked or degraded.	Design amplicons to be as small as possible (<100 bp).	DNA isolated using Covaris AFA technology is of the highest possible quality. Some FFPE sample blocks may be too degraded or cross-linked for some applications.

APPENDIX C – DRY BLOCK HEATER CALIBRATION PROCEDURE

1. Place the Covaris Centrifuge and Heat Block microTUBE adapter into the dry block heater.
2. Add water to the separate hole in the Heat Block adapter and insert a glass thermometer.
3. Set the dry block heater temperature to 60˚C.
4. Wait for the dry block heater to reach the set point.
5. Check temperature displayed by the thermometer ($\mathbf{T_{th}}$).
6. If $\mathbf{T_{th}}$ is between 59˚C and 61˚C (setpoint +/- 1 ˚C), use 60˚C for $\mathbf{Tset_1}$.
7. Otherwise, use the formula below to obtain $\mathbf{T\ set_1}$:

$\mathbf{T\ set_1}$ (˚C) = 120˚C- $\mathbf{T_{th}}$

8. Repeat steps 3-7 with an initial set point of 90˚C to obtain $\mathbf{T\ set_2}$:

$\mathbf{T\ set_2}$ (˚C) = 180˚C- $\mathbf{T_{th}}$

APPENDIX D – OPTIONAL DNASE TREATMENT OF ISOLATED RNA

This protocol isolates total RNA that contains small amounts of genomic DNA. Two optional DNase treatment protocols are provided here if DNA-free RNA must be isolated:

1. Optional in-solution DNase digestion with TURBO DNA-free kit:

   Based on the manufacturer, the TURBO DNA-free™ kit is designed to remove contaminating DNA from RNA preparations, and to subsequently remove the DNase and divalent cations from the sample. The TURBO™ DNase in the kit is an engineered version of wild type DNase I with much greater catalytic efficiency. **This treatment must be performed after step 11 in the RNA purification section** according to the manufacturer's protocol provided in the kit.

   The kit does not have an extensive procedure to purify RNA after the treatment. It uses a DNase inactivation reagent to inactivate DNase and to remove divalent cations, such as magnesium and calcium in the DNase treatment reactions. It is simple to use, however the inactivation reagent may inhibit reverse transcription at high concentration. Therefore the TURBO DNA-free™ treated RNA should comprise of less than 20% of the total RT-PCR reaction volume.

   An additional optional on-column DNase digestion protocol provided below can be used if this is a concern.

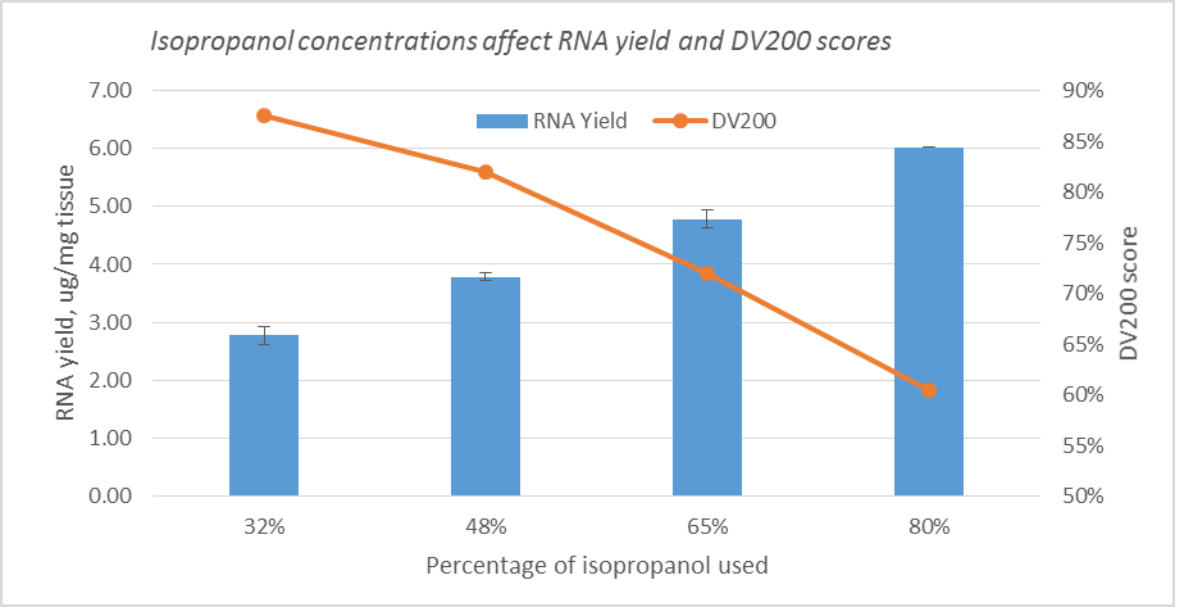
2. Optional on-column DNase digestion with DNase from TURBO DNA-free kit:

In this protocol, the DNase from the TURBO DNA-free kit is used. However, any commercially available RNase-free DNase with appropriate buffer may work as well.

1. Place the RNA Columns into new collection or 2.0 ml microcentrifuge tubes after the 1st wash of **step 8** in Section 3 - RNA purification.
2. Prepare a 1 X TURBO DNase mixture on the RNA Purification column:
    - RNase-free H_2O, 88 µl
    - 10X TURBO DNase buffer, 10 µl
    - TURBO DNase, 2 µl
3. Close cap and vortex to mix.
4. Incubate at room temperature for 20-30 minutes.
5. Add 175 µl B1 buffer and 300 µl 65% isopropanol per Column.
6. Spin at 11, 000 x g for 30 seconds.
7. Pipette the flow-through that is present in the collection tube back into the Column.
8. Spin at 11, 000 x g for 30 seconds.
9. Discard the flow-through and place the Purification Column back onto the Collection Tube.
10. Proceed with **Step 8** of the RNA purification protocol (Section 3).

APPENDIX E – ISOPROPANOL CONCENTRATION AND DV200 SCORES

The isopropanol concentration used in the Section 3 - RNA purification will impact RNA yield and size distribution (as expressed by DV200 score [2]). If high DV200 scores are desirable, use less concentrated isopropanol. However, if maximum RNA yield is desired at the expense of the DV200 score (increase of <200nts RNA fraction), use more concentrated isopropanol. The effects on FFPE RNA yield and DV200 score can be seen in the Figure below.



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.

ADDITIONAL NOTES

1. Covered by US Patent 9,080,167
2. Other patents pending
3. Best Practices for determining the yield and purity of isolated DNA:
   - To determine DNA yield with the highest level of accuracy, a fluorometric assay such as Qubit ™ (Life Technologies) should be used.
   - In addition, spectrophotometric analysis of DNA for A260/280 and A260/230 ratios will determine if protein or peptide/salt contamination is present in the sample.
4. Tissue Blocks were obtained from: Theresa Kokkat, PhD and Diane McGarvey, Cooperative Human Tissue Network (CHTN), Eastern Division, University of Pennsylvania, USA
5. See following link: http://covaris.com/resources/protocols/ for updates to this document.
6. 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.