PROTOCOL

Covaris

truXTRAC™ FFPE microTUBE RNA Kit – Column Purification (25)

Adaptive Focused Acoustics™ (AFA) -based RNA extraction and purification from Formalin-Fixed, Paraffin-Embedded Tissue using columns

Product PN 520161

Contents

INTENDED USE

The truXTRAC FFPE RNA Kit is intended for use in molecular biology research applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

INTRODUCTION

The truXTRAC FFPE RNA Kit is designed for the controlled and efficient extraction of RNA from Formalin Fixed, Paraffin Embedded (FFPE) tissue samples with the Covaris Adaptive Focused Acoustics (AFA™). AFA enables the active removal of paraffin from FFPE tissue samples in 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. The AFA process enables the use of thicker sections, which can increase RNA yield and minimize the impact of increased RNA degradation at the exposed surfaces of a section. The truXTRAC process results in high yields of high-quality RNA well suited for analytical methods such as next-generation sequencing or qPCR.

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

Important Notes on FFPE Samples:

The yield of RNA from FFPE tissue blocks is highly variable. Factors such as fixation time, size and thickness of the sections, the ratio of tissue to wax, the type of tissue, and the age of the FFPE block are the main causes for this variability.

The quality of RNA isolated from FFPE samples is also highly variable. During the fixation process, RNA is cross-linked to proteins and other nucleic acid molecules to varying degrees. Incomplete reversal of this crosslinking may cause the isolated RNA to perform less well in downstream applications such as PCR and qPCR. In addition, the size of RNA fragments isolated from FFPE samples is generally smaller than that of RNA isolated from fresh or frozen tissues. 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 RNA 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 kit. Ideally, samples should be extracted immediately after sectioning.

REVISION HISTORY

Part Number	Revision	Date	Description of change
010268	A	11/14	Initial release
010268	B	03/15	Updated M220 Holder requirement
010268	C	05/15	Changed name of Heat Block microTUBE Adapter
010268	D	07/15	Sample input requirements clarified
010268	E	09/15	Changed incubation time to reverse formaldehyde crosslinks to 1 hour
010268	F	05/16	Update product name, add calibration procedure, clarify preparation procedure
010268	G	01/18	Change LE220 rack and water level
010268	H	8/19	Removed tissuePICK, sectionPICK and related accessories

KIT CONTENTS

RNA Lysis	3 ml
B1 Buffer	7.5 ml
RNA Wash	11 ml
RNA Elution	3 ml
PK Solution	300 µl
DNase I (Lyophilized)	1 Vial
DNase Buffer	220 µl
MnCl2 Solution	400 µl
RNA Purification Columns	25
RNA Collection Tubes	25
RNA Elution Tubes	25
microTUBE-130 AFA Fiber Pre-Slit Screw-Cap	25

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

STORAGE

This kit should be stored at room temperature (18 – 25 °C).

The reconstituted DNase I should be stored at (–20°C).

SUPPLIED BY USERS

Covaris Instruments and Parts

Required parts						
Focused-ultrasonicator	**LE220**	**E220 & E210**	**E220 evolution**	**S-Series**	**M220**	**ME220**
Rack/ Holder	Rack 24 Place microTUBE Screw-Cap PN500308	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)					

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

Other supplies:

- Microcentrifuge with 16,000 x g capability
- Water bath, oven or dry block heater (e.g., Eppendorf ThermoMixer) for 1.5 or 2 mL tubes, capable of heating to 80°C.
- Ethanol (>96%), MB Grade e.g., Thermo Scientific (PN BP2818-100).
- 1.5 mL nonstick nuclease free microfuge tubes e.g., Life Technologies (PN AM12450).
- Nuclease Free water, e.g., Life technologies (PN AM9932) or equivalent.

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, both for increased yield and that DNA or RNA in the exposed surfaces of a section tends to degrade quickly. **We recommend 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. We strongly advise trimming off any excess of 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" or "curls"	
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	Scalpel or razor blade	Scalpel or razor blade	NA	
Maximum number of samples Per Tube*	2	2	2*	1*

* Numbers represent trimmed sections only

NOTES

A. If the FFPE sample is longer than about 10 mm, cut it in half before loading.

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 RNA extracted from FFPE tissue blocks is highly dependent on tissue

collection and paraffin embedding procedures. For good yields of high quality RNA:

- Use a maximum fixation time of 24 hours
- Use Formalin solution, neutral buffered, 4%
- Fix sample tissue sample as quickly as possible after collection

Buffers

1. **Add 275 µL of Nuclease Free water to DNase I bottle.** Mix by inverting bottle until pellet is completely dissolved. Do not vortex. We recommend storing the rehydrated DNase into aliquots using sterile, RNase-free microcentrifuge tubes.
   Store reconstituted DNase I at –20°C.

2. **Add ethanol to RNA Wash**: Add 25 ml EtOH to 11 ml RNA Wash buffer before use.

3. **Check Buffer B1 and RNA Lysis Buffer:** A white precipitate may form during storage. Incubate the bottles at 50 – 70 °C before use to dissolve any precipitate.

Focused-ultrasonicator

- For S, E, or LE-Series Focused-ultrasonicators, set up the instrument as shown in table. Wait for the water to reach temperature and to degas.
- For the M220 Focused-ultrasonicators, put the Holder PN500414 and the Insert PN500489 (or the discontinued Holder PN500358 without insert) in place and fill the water bath until the water reaches the top of the holder. Allow system to reach temperature (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.

Focused-ultrasonicator setup

Instrument	Water level*	Chiller temp	Intensifier PN500141	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
LE-Series	**15**	**18°C**	**NA**	500308 Rack 24 Place microTUBE Screw-Cap	PN500308

***** 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 detailed instructions on how to prepare your instrument please refer to the User Manual.

Heating Blocks, Water Baths, or Ovens

Preheat dry block heaters, water baths, or ovens to 56°C (or **T set$_1$** - see Appendix E) and 80°C (or **T set$_2$** - see Appendix E).

When using a dry block heater, microTUBEs need to be placed into microTUBE Adapters (PN500406) first.

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

2 - RNA EXTRACTION FROM FFPE TISSUE

1. Open microTUBE Screw-Cap, add 110 µl RNA Lysis Buffer into microTUBE and load FFPE tissue. Affix Screw-Cap back in place.

> **NOTE***:* if the FFPE tissue samples are loose or broken the samples may be added to the microTUBE prior to RNA Lysis Buffer addition to facilitate easier loading.

2. Process the samples using the settings provided in Table 1 below to dissociate the paraffin and rehydrate the tissue. (Please see the example in Appendix A.)
   During the AFA process it is normal for the solution to turn milky white as the paraffin is emulsified.

Table 1 - Paraffin removal and tissue rehydration settings

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)	**15%**	**450 Watts**	**200**	**300 sec**	**20 °C**

(1) As Covaris LE220 process multiple samples at a time, its PIP is distributed across microTUBEs, and power received by individual microTUBEs stays within the 200 W limit.

3. Open Screw-Cap microTUBE, add 10 µl of PK solution to the sample and affix Screw-Cap back in place.

4. Process the sample using the settings provided in Table 2 below to properly mix Proteinase K with the sample.

Table 2 – Proteinase K mixing 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[(1)]	**30%**	**450 Watts**	**200**	**10 sec**	**20 °C**

5. **Proteinase K digestion at 56°C** (or **T set$_1$** - see Appendix C)**:** Incubate samples for 15 minutes at 56°C for proteinase K digestion. **When using a dry block heater, microTUBEs need to be placed into microTUBE Adapters first.**

> **NOTE**: Some tissue types, such as fibrous or muscle tissues, may require longer lysis incubations to maximize RNA recovery (i.e., 2 hours to overnight). Incubation time for these types of tissues should be determined empirically or contact Covaris Application Support.

6. **Crosslink reversal at 80°C** (or **T set$_2$** - see Appendix C)**:** Incubate samples for 1 hour at 80°C to reverse formaldehyde crosslinks. **When using a dry block heater, microTUBEs need to be placed into microTUBE Adapters first.**

> **NOTE**: If you are using the same heat block for both the 56°C & 80°C incubations, the microTUBE should be stored at room temperature until the heat block reaches 80°C

7. Transfer the sample to a clean 1.5 ml microcentrifuge tube and centrifuge at 15,000 x g for 15 minutes.

8. Transfer the supernatant to a new microcentrifuge tube taking care to leave the pellet and residual wax behind. Small amounts of residual wax will not interfere with the RNA purification).

9. **Skip to Section 3 – RNA Purification if DNase I treatment is not required.**

10. DNase I treatment (Optional): The sample can be treated with DNase I to remove residual DNA before RNA purification. Prepare the DNase master mix as shown below.

Component	Volume Per Sample (in µl)
MnCl2 Solution	**13**
DNase Buffer	**7**
DNase I	**10**
Total Volume per sample; 30 µl	

> **NOTES** – DNase I usage:
> - Prepare only the amount of DNase master mix required
> - Thaw and keep the DNase I enzyme on ice during use
> - The DNase I enzyme is sensitive to physical inactivation. Mix by gentle pipetting. Do not vortex.
> - Prepare the DNase treatment mix immediately before use. The components of the DNase master mix should be stored separately and mixed fresh for each set of RNA extractions.

11. Add 30 µl of freshly prepared DNase master mix to each sample and mix by pipetting gently.
12. Incubate for 15 minutes at room temperature (20–25°C)
13. Proceed to Section 3 **immediately** – RNA Purification.

3 - RNA PURIFICATION

1. If DNase I treatment **was not** conducted add 30 µl of nuclease free water to each sample.
2. Add 175 µl Buffer B1 to your samples and vortex to mix.
3. Add 200 µl ethanol (>96%) to samples and vortex to mix.
4. Insert RNA Purification Columns into provided RNA Collection Tubes.
5. Transfer the samples to the RNA Purification Columns. *Note*: Small amounts of residual wax will not interfere with the RNA purification).
6. Spin the assemblies (RNA Purification columns/collection tubes) at 14,000 x g for 1 minute.

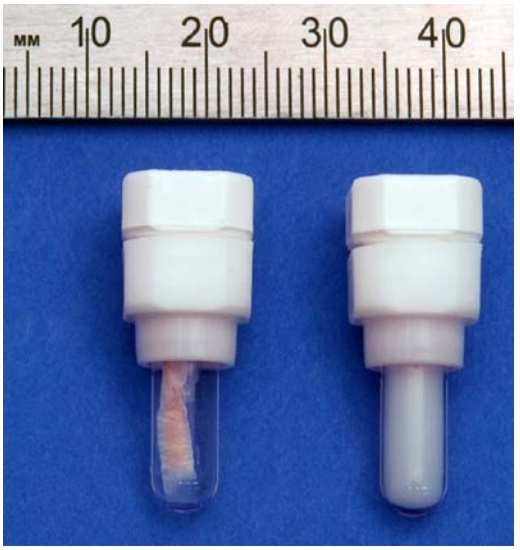
> **NOTE:** If all the sample did not pass through the column, please add an additional 5 minutes spin at 14,000 x g

7. Discard the flow-through and place the RNA Purification Column back in the Collection Tube.
8. **1st wash**: Add 500 µl RNA Wash. Spin the assemblies at 14,000 x g for 1 minute.
9. Discard the flow-through and place the Purification Columns back in the Collection Tubes.
10. **2nd wash**: Add 500 µl RNA Wash. Spin the assemblies at 14,000 x g for 1 minute.
11. Discard the flow-through and place the RNA Purification Columns in the RNA Collection Tubes.
12. **Dry RNA Purification Column**: Spin the assemblies at 14,000 x g for 2 minutes.
13. **Elute RNA: RNA elution is performed in two steps:**
    Step 1: Place the RNA Purification Columns into a RNA Elution Tubes and add 20 to 50 µl RNA Elution Buffer to the center of the column. Spin the assemblies at 200 x g for 2 minutes.
    Step 2: Without removing the assemblies from the centrifuge spin at 14,000 x g for an additional 1 minute.
14. Remove assemblies from the centrifuge, discard RNA Purification Columns and keep the eluted RNA on ice for further analysis. RNA should be kept at -80°C for long term storage.

> **NOTE:** RNA Elution buffer composition is Nuclease Free Water

APPENDIX A – PARAFFIN EMULSIFICATION WITH AFA ENERGY

Paraffin is emulsified in microTUBE Screw-Cap using a Covaris S220 Focused-ultrasonicator. Sample before (left side) and after (right side) processing. Sample was a 10 µm kidney tissue section.



APPENDIX B – TROUBLESHOOTING GUIDE

Issue	Cause	Solution	Comments / Suggestions
Low yield of RNA	Low tissue to wax ratio in FFPE section.	We strongly advise trimming off any excess of 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.	Excess paraffin will adversely affect the yield and quality of DNA and RNA extracted from FFPE. In your initial use of the truXTRAC FFPE kit use FFPE blocks that have been well characterized for yield and quality.
	Insufficient tissue input	Increase FFPE tissue section thickness or use more sections up to 5mg total weight.	
No RNA	Ethanol not added to buffer RNA Wash.	Repeat the procedure with fresh samples and ensure ethanol is added to RNA Wash.	
No RNA	Step 4 has been omitted.	Repeat the procedure with appropriate mixing.	Step 4 mixes the Proteinase K with the sample. Proteinase K Buffer contains Glycerol, and may fall to the bottom of the microTUBE. Be sure to mix appropriately.
RNA concentration is too low	Elution volume is too high.	Repeat procedure using lower elution volume (20 µl minimum volume is required). Alternatively, Concentrate samples using ethanol precipitation or other means	
RNA does not perform well in downstream applications such as qPCR	RNA in FFPE sample blocks is severely cross-linked or degraded.	Design amplicons to be as small as possible (<100 bp).	RNA 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 – HEATING SOURCE CALIBRATION PROCEDURE

1. If using a dry block heater, place the Covaris Heat Block microTUBE Adapters into the heating block.
2. Add water to one of the Heat Block microTUBE Adapters and insert a glass thermometer or place a glass thermometer into the water bath or the oven.
3. Set the heating source temperature to 56˚C.
4. Wait for the heating source to reach the set point.
5. Check temperature displayed by the thermometer (**Tth**).
6. If **Tth** is between 55˚C and 57˚C (setpoint +/- 1 ˚C), use 56˚C for **$Tset_1$**.
7. Otherwise, use the formula below to obtain **$T\ set_1$**:

$T\ set_1$ (˚C) = 120˚C- **Tth**

8. Repeat steps 3-7 with an initial set point of 80˚C to obtain **$T\ set_2$**:

$T\ set_2$ (˚C) = 160˚C- **Tth**

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 RNA yield with the highest level of accuracy, a fluorometric assay such as Qubit ™ (Life Technologies) should be used.
   - In addition, spectrophotometric analysis of RNA 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://covarisinc.com/wp-content/uploads/pn_010268.pdf** 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.