Advanta FFPE RNA Extraction Kit

Contents

About This Protocol

This protocol describes how to extract RNA from formalin-fixed, paraffin-embedded (FFPE) samples using the Advanta™ FFPE RNA Extraction Kit.

IMPORTANT Before using the reagent kit, read and understand the detailed instructions and safety guidelines in this document. For complete safety information, see Appendix B.

Safety Alert Conventions

Fluidigm documentation uses specific conventions for presenting information that may require your attention. Refer to the following safety alert conventions.

For hazards associated with chemicals, this document follows the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and uses indicators that include a pictogram and a signal word that indicates the severity level:

Indicator	Description
	Pictogram (see example) consisting of a symbol on a white background within a red diamond-shaped frame. Refer to the individual safety data sheet (SDS) for the applicable pictograms and hazards pertaining to the chemicals being used.
DANGER	Signal word that indicates more severe hazards.
WARNING	Signal word that indicates less severe hazards.

This document also uses the following indicators for presenting information that may require your attention:

Indicator	Description
	Pictogram (see example) consisting of a symbol on a white background within a black triangle-shaped frame.
CAUTION	Signal word that indicates a potentially hazardous situation that could result in minor or moderate personal injury if not avoided.
IMPORTANT	Signal word that indicates information necessary for proper use of products or successful outcome of experiments.

Safety Data Sheets

Read and understand the SDSs before handling chemicals. To obtain SDSs for chemicals ordered from Fluidigm, either alone or as part of this system, go to fluidigm.com/sds and search for the SDS using either the product name or the part number.

Some chemicals referred to in this user guide may not have been provided with your system. Obtain the SDSs for chemicals provided by other manufacturers from those manufacturers.

Introduction

The Advanta FFPE RNA Extraction Kit provides a rapid and highly efficient xylene-free method for the extraction of total RNA from archived FFPE samples. A volume of up to 2.0 mm^3 of tissue sections (curls or slides) can be processed in each reaction, and the entire procedure can be completed in about 3 hours for one sample. Using 1 kit, you can process a total of 50 samples. RNA extracted from FFPE samples can be analyzed for quality and quantity using absorbance measurement, dye-binding measurement, capillary electrophoresis, or any other preferred method.

Workflow Overview

Extracting RNA from FFPE samples consists of the following steps:

1	2	3	4	5
Deparaffinize samples.	Perform Proteinase K digestion.	Perform bead cleanup.	Perform DNase I digestion and bead cleanup.	Quantify and qualify RNA.

The time needed to extract RNA depends on the number of samples you are processing. If the number of samples is large, prepare them in batches of 8–20.

Number of Samples	Total Time*
1	2 hr 40 min
8	5 hr 30 min
20	6 hr 30 min

* Includes a 1-hour incubation step.

Materials

Required Reagents

IMPORTANT Store reagents according to manufacturer's storage recommendations as soon as you receive them.

Fluidigm Provided Reagents

Reagent	Source	Part Number	Storage
Advanta FFPE RNA Extraction Kit*	Fluidigm	101-6773	
• Advanta FFPE RNA Extraction Kit—50 Rxn			–20 °C
• Advanta FFPE Purification Beads Kit			4 °C

* For a list of the reagent kit components, see Appendix A.

Reagents from Other Suppliers

Product Name	Source	Part Number
Hexadecane*	Sigma-Aldrich	H6703
Ethyl alcohol (ethanol), Pure, 200 proof, or equivalent	Sigma-Aldrich	E7023

* Dispose of hexadecane waste appropriately.

Required Consumables

Product	Source	Part Number
1.5 mL microcentrifuge tubes	Major laboratory supplier (MLS)	—

Required Equipment

Product	Source	Part Number
DynaMag™-2 or equivalent magnetic separator for 1.5 mL tubes	Thermo Fisher Scientific	12321D
Two heating blocks for 1.5 mL tubes	Major laboratory supplier (MLS)	—
Microcentrifuge capable of 15,000 x *g*, with rotor for 1.5 mL tubes	MLS	—
Mini centrifuge compatible with microcentrifuge tubes	MLS	—
Vortexer	MLS	—
Pipettes (P20, P200, P1000) and appropriate low-retention filtered tips	MLS	—

Sample Requirements

This protocol is applicable for extraction of FFPE tissue sections that are 5–10 µm thick and have an area of 20 to 200 mm^2, for a total volume of up to 2.0 mm^3 of tissue in each reaction. Due to variations in the composition of different tissues and sample types, we recommend that you test input amounts for each tissue type and that you start with the smallest volume possible. Too much tissue in the extraction reaction will adversely affect the RNA yield.

This protocol is compatible with both slides and curls.

Before You Begin

IMPORTANT Read and understand the safety information in Appendix B.

To ensure reliable results:

- Ensure that lab consumables (tubes, tips) used for the RNA handling steps are RNase-free.
- Use only the reagents provided in the required kit. Do not make substitutions.
- Thaw reagents at room temperature (15–30 °C) unless directed otherwise.
- Mix and centrifuge reagents as directed.
- Allow enough time for the heat blocks to heat up for the incubation steps. Two heating blocks are preferred (55 ˚C and 90 ˚C).
- Because ethanol is a hygroscopic substance, be sure to cap each tube right after pipetting ethanol into it.

RNA Extraction from FFPE Samples

Retrieve the Reagents

Required Reagent	Preparation
DNase I (PN 101-6446)	Remove from –20 °C right before use. Keep on ice if needed for long periods.
Proteinase K (PN 101-6447)	
FFPE Extraction Buffer (PN 101-6441)	Remove from –20 °C, thaw, and keep at room temperature.
RNA Enhancer (PN 101-6443)	
FFPE Purification Reagent (PN 101-6442)	
DNase Buffer (PN 101-6444)	
EDTA (PN 101-6445)	
Dilution Reagent (PN 100-8725)	
AMPure® XP Beads (PN 101-5998)	Remove from 4 °C and keep at room temperature.
FFPE samples	Remove from storage and keep at recommended temperature.
Hexadecane	Remove from storage and keep at room temperature. **NOTE** If hexadecane was previously frozen, thaw completely before use.

IMPORTANT
- Centrifuge the enzymes before use.
- Vortex and centrifuge all other buffers and reagents before use.

Extract RNA

1. Prepare 80% ethanol fresh for each use. The total volume of 80% ethanol needed per sample is 2,000 µL (including approximately 30% overage). The following recommendation for preparing the 80% ethanol is for one sample. Scale up appropriately.
   a. Add 400 µL of PCR-grade water to a 15 mL centrifuge tube.
   b. Add 1,600 µL of 100% ethanol. Mix by vortexing or inverting.
   c. Cap the tube and store at room temperature.
2. Add FFPE tissue samples (curls or slides, up to 2.0 mm^3 volume) to a 1.5 mL tube.

   NOTE If using FFPE slides, carefully transfer the FFPE samples from the glass slides into a 1.5 mL tube with a scalpel or blade.
3. Add 1 mL of hexadecane to each 1.5 mL tube containing FFPE samples. Vortex the tubes at maximum speed for 10 seconds and then centrifuge for 2 seconds to collect contents.
4. Incubate tubes at 55 °C for 3 minutes to completely melt the paraffin.
5. Centrifuge the tubes at 15,000 x *g* for 2 minutes at room temperature to pellet the material. Depending on the tissue input, a pellet might not be visible.
6. Remove and discard the supernatant by pipetting, removing as much as possible but being careful not to disturb the pellet:
   a. Use a P1000 pipette to remove 800 µL.
   b. Use a P200 pipette to remove 180 µL.
   c. Use a P20 pipette to remove the rest, if necessary.
7. Add 1 mL of 100% ethanol to each tube and mix by vortexing at maximum speed for 10 seconds. Centrifuge at 15,000 x *g* for 2 minutes at room temperature to ensure that the contents are collected at the bottom of the tube.
8. Remove and discard the supernatant by pipetting. Do not remove any of the pellet.
   a. Use a P1000 pipette to remove 800 µL.
   b. Use a P200 pipette to remove 180 µL.
   c. Use a P20 pipette to remove the rest, if necessary.
9. Air-dry the pellet at room temperature for 10 minutes until all residual ethanol has evaporated.

10 Into each tube, add FFPE Extraction Buffer, RNA Enhancer, and Proteinase K using the volumes shown:

Component	Volume (µL)
FFPE Extraction Buffer	100
RNA Enhancer	4
Proteinase K	4
Total	**108**

NOTE You can scale up volumes to make a master mix for this step.

11 Mix by vortexing the tubes at medium speed for 5 seconds, and then centrifuge for 2 seconds to collect contents.

12 Incubate the tubes at 55 °C for 1 hour. After incubation, vortex the tubes at medium speed for 5 seconds, and then centrifuge for 2 seconds to collect contents. Reset the heating block temperature to 37 °C.

13 Incubate the tubes at 90 °C for 10 minutes.

14 While the tubes are incubating, prepare the mixture for the bead cleanup:

a Resuspend the AMPure XP Beads by vortexing the stock bottle of beads at maximum speed for 20 seconds.

b In a new set of tubes, prepare the bead mixture as shown:

Component	Volume (µL)
AMPure XP Beads	78
FFPE Purification Reagent	62
Total	**140**

c Label the bead mixture tubes accordingly and save them for the bead cleanup procedure.

NOTE You can scale up volumes to make a master mix for this step.

15 After incubation in Step 13, cool the tubes to room temperature, vortex the sample tubes briefly, and then centrifuge at 15,000 x *g* for 2 minutes at room temperature. Reset the heating block temperature to 75 °C.

Perform Bead Cleanup

1 Transfer 100 µL of the supernatant to each of the tubes containing beads that were prepared in step 14 of the previous section. Mix well by pipetting up and down three times. Discard the tubes that contained the pellets.

2 Vortex the bead mixture tubes at medium speed for 10 seconds, and then centrifuge for 2 seconds to collect contents.

3 Incubate the tubes at room temperature for 10 minutes.

4 Place the tubes on a magnetic separator for 2 minutes or until the solution is clear.

5 Without disturbing the beads, and keeping the tubes on the magnetic separator, use a pipette to remove and discard the supernatant. Leave ~5 µL of the supernatant in the tube to avoid drawing out beads.

6 Wash the beads three times with 80% ethanol:

   a Keeping the tubes on the separator, add 400 µL of 80% ethanol to each tube.

   b Incubate the tubes at room temperature for 1 minute.

   c Without disturbing the beads, and keeping the tubes on the magnetic separator, remove and discard all of the ethanol.

   d Repeat Steps 6a–6c twice for a total of three washes.

7 Remove the remaining ethanol by pipetting using a 20 µL tip. Air-dry the beads at room temperature for 10 minutes (or dry at 37 °C for 1 minute) to allow residual ethanol to evaporate.

8 Prepare the eluate:

   a Transfer the tubes to a rack and add 20 µL of Dilution Reagent to each tube. Flick or gently snap the bottom of the tube with your forefinger to mix the contents.

   NOTE If the beads do not resuspend easily, pipet the solution up and down until the beads are completely resuspended.

   b Vortex the tubes at medium speed for 5 seconds and then centrifuge for 2 seconds to collect contents.

   c Incubate the tubes at room temperature for 2 minutes.

   d Place the tubes on a magnetic separator for 2 minutes or until the solution is clear.

9 Without disturbing the beads, and keeping the tubes on the magnetic separator, transfer each eluate (~17 µL) to a new 1.5 mL tube for DNase I digestion.

IMPORTANT Leave ~3 µL of eluate in the tube to avoid drawing out beads.

Perform DNase I Digestion

1 To each of the eluate tubes, add 2 µL of 10X DNase reaction buffer and 1 µL of DNase I, and then vortex at medium speed for 5 seconds, and then centrifuge for 2 seconds to collect contents.

IMPORTANT We do not recommend making a master mix with the DNase reaction buffer and DNase I for this step.

2 Incubate the tubes at 37 °C for 10 minutes. Centrifuge for 2 seconds to collect contents.

3 Add 2 µL of 50 mM EDTA to each tube. Vortex each tube at medium speed for 5 seconds, and then centrifuge for 2 seconds.

4 Incubate at 75 °C for 10 minutes. After incubation, transfer the tubes to a rack and allow them to cool to room temperature for 2 minutes. Centrifuge the tubes for 2 seconds to collect contents.

5 Add 9 µL of FFPE Purification Reagent to each of the tubes.

6 Vortex the stock bottle of AMPure XP Beads at maximum speed for 20 seconds, and then add 22 µL of the beads to each tube. Flick or gently snap the bottom of the tubes to mix the contents, and then vortex the tubes at medium speed for 10 seconds. Centrifuge the tubes for 2 seconds to collect contents.

7 Incubate the tubes at room temperature for 10 minutes.

8 Place the tubes on a magnetic separator for 2 minutes or until the solution is clear. Without disturbing the beads, and keeping the tubes on the magnetic separator, use a pipette to remove and discard the supernatant. Leave ~5 µL of the supernatant in the tube to avoid drawing out beads.

9 Wash the beads three times with 80% ethanol:

   a Keeping the tubes on the separator, add 100 µL of 80% ethanol to each tube to wash the beads.

   b Incubate the tubes at room temperature for 1 minute.

   c Without disturbing the beads, and keeping the tubes on the magnetic separator, remove and discard the ethanol.

   d Repeat steps 9a–9c twice for a total of three washes.

10 Remove the remaining ethanol by pipetting. Air-dry at room temperature for 10 minutes (or dry at 37 °C for 1 minute) to allow residual ethanol to evaporate.

11 Prepare the eluate:

   a Transfer the tubes to a rack and add 20 µL of Dilution Reagent to each tube. Flick or gently snap the bottom of the tube with your forefinger to mix the contents.

   NOTE If the beads do not resuspend easily, pipet the solution up and down until the beads are completely resuspended.

   b Vortex the tubes for 5 seconds at medium speed, and then centrifuge for 2 seconds to collect contents.

   c Incubate the tubes at room temperature for 2 minutes.

   d Place the tubes on a magnetic separator for 2 minutes.

12 Without disturbing the beads, and keeping the tubes on the magnetic separator, transfer the eluate (~18 µL) to a new 1.5 mL tube.

   IMPORTANT Leave ~2 µL of eluate in the tube to avoid drawing out beads.

13 Proceed with quantification/qualification immediately or store RNA samples at –20 °C for up to 7 days or at –80 °C for longer-term storage.

Quantify and Qualify RNA

Extracted RNA from FFPE samples can be evaluated for quality and concentration using methods based on absorbance (such as NanoDrop™), dye-binding (Qubit®), and capillary electrophoresis (such as Bioanalyzer®). More recently, DV_{200} has been used as a qualitative metric for evaluating the integrity of RNA extracted from FFPE-derived samples.[1] Both DV_{200} and RNA integrity number (RIN) metrics can be obtained using the Agilent® Bioanalyzer. RIN values are automatically output for each RNA sample that is analyzed using an RNA assay on Bioanalyzer. For DV_{200} calculation, use either the RNA 6000 Nano or Pico kit from Agilent, depending on the most typical RNA concentration obtained. These kits have different qualitative ranges for DV_{200} calculation, so be certain that your RNA samples are within the qualitative range of each kit before you start your assay. NanoDrop RNA concentration can be used to calculate the most appropriate dilution factor for the samples. When running the RNA Nano or Pico assay on Bioanalyzer, be certain to select the **Eukaryote Total RNA assay** for evaluation of the full range of RNA molecule sizes.

The percentage of RNA fragments of >200 nucleotides (DV_{200}) can be calculated from a Bioanalyzer trace by performing a smear analysis as follows:

1. Select one of the samples in the data file for the electropherogram view and under the Global tab, change Normal to **Advanced**.
2. Select the checkbox for **Smear Analysis**.
3. Click **Table**, add a region, and enter **200–10,000 nucleotides** (or adjust 10,000 to maximum size possible) in the popup window. Name this region **DV200** and click **OK**.
4. Select the Region Table tab in the trace window to display the results. The DV_{200} value will be displayed in the % of Total column (Figure 1).

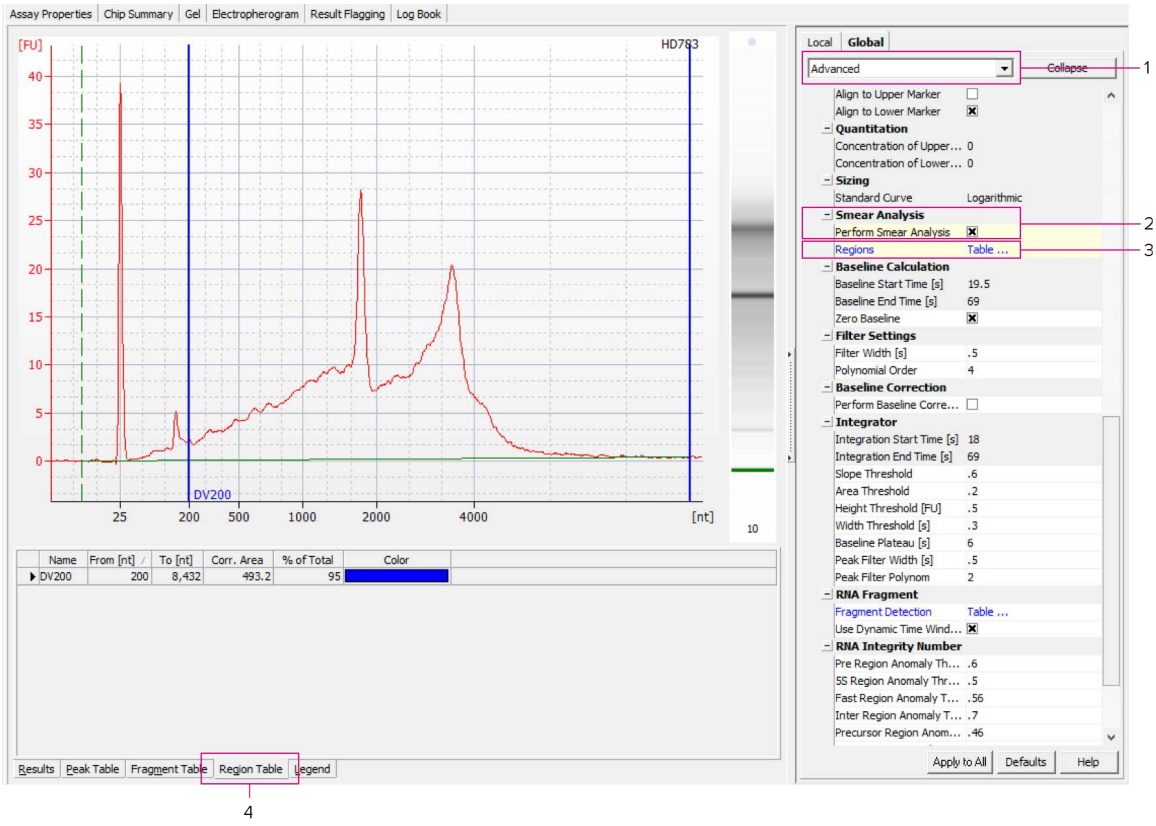


Figure 1. Bioanalyzer trace. Agilent RNA 6000 Pico kit was used for the procedure, and the Eukaryote Total RNA assay was selected to run the instrument for the DV_{200} analysis.

Reference

1. Eikrem, O. et al. “Transcriptome sequencing (RNAseq) enables utilization of formalin-fixed, paraffin-embedded biopsies with clear cell renal cell carcinoma for exploration of disease biology and biomarker development.” *PloS One* 11(2) (2016): e0149743.

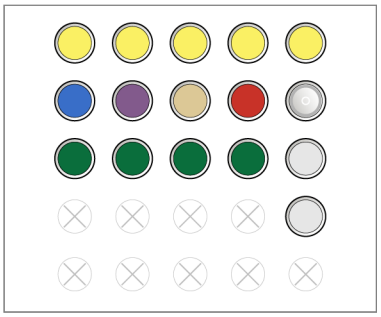
Additional References

Landolt, L. et al. “RNA extraction for RNA sequencing of archival renal tissues.” *Scandinavian Journal of Clinical and Laboratory Investigation* 76(5) (2016): 426–34.

Von Ahlfen, S. et al. “Determinants of RNA quality from FFPE samples.” *PloS One* 2(12) (2007): e1261.

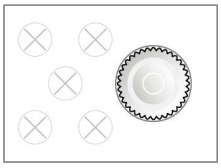
Appendix A: Advanta FFPE RNA Extraction Kit Components (PN 101-6773)

Advanta FFPE Extraction Kit – 50 Rxn (PN 101-6449)



- 5 tubes, FFPE Extraction Buffer, 1.0 mL (PN 101-6441)
- 1 tube, RNA Enhancer, 250 µL (PN 101-6443)
- 1 tube, Proteinase K (800 units/mL), 200 µL (PN 101-6447)
- 4 tubes, FFPE Purification Reagent, 1.0 mL (PN 101-6442)
- 1 tube, DNase I (2 units/µL), 50 µL (PN 101-6446)
- 1 tube, DNase Buffer (10X), 100 µL (PN 101-6444)
- 1 tube, EDTA (50 mM), 100 µL (PN 101-6445)
- 2 tubes, Dilution Reagent, 1.7 mL (PN 100-8725)

Advanta FFPE Purification Beads Kit (PN 101-6721)



- 1 bottle, AMPure XP Beads, 5 mL (PN 101-5998)

Appendix B: Safety

General Safety

In addition to your site-specific safety requirements, Fluidigm recommends the following general safety guidelines in all laboratory and manufacturing areas:

- Use personal protective equipment (PPE): safety glasses, fully enclosed shoes, lab coats, and gloves.
- Know the locations of all safety equipment (fire extinguishers, spill kits, eyewashes/showers, first-aid kits, safety data sheets, etc.), emergency exit locations, and emergency/injury reporting procedures.
- Do not eat, drink, or smoke in lab areas.
- Maintain clean work areas.
- Wash hands before leaving the lab.

Chemical Safety

Wear personal protective equipment (gloves, safety glasses, fully enclosed shoes, lab coats) when handling chemicals.

Do not inhale fumes from chemicals. Use adequate ventilation and return caps to bottles immediately after use.

Check regularly for chemical spills or leaks. Follow SDS recommendations for cleaning up spills or leaks.

Disposal of Products

Used reagents should be handled and disposed of in accordance with federal, state, regional, and local laws for hazardous waste management and disposal.

For technical support visit fluidigm.com/support.

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