July 2023

EZ2® AllPrep® DNA/RNA FFPE Handbook

EZ2 AllPrep DNA/RNA FFPE Kit

For automated simultaneous purification of genomic DNA and RNA from formalin-fixed, paraffin-embedded (FFPE) tissues using the EZ2 Connect instruments

Sample to Insight

Contents

Kit Contents

Kit name Catalog no. No. of reactions	EZ2 AllPrep DNA/RNA FFPE Kit 954734 48
Buffer ATL	14 mL
Buffer PKD	15 mL
DNase Booster Buffer	2 mL
Paraffin Removal Solution	2 x 20 mL
Proteinase K	2 x 1.25 mL
RNase-Free DNase I (lyophylized)	1500 units*
RNase-Free Water (for use with RNase-free DNase I)	1.9 mL
EZ2 AllPrep DNA/RNA FFPE Cartridges†‡	48
Disposable Tip Holders	2 x 50
Disposable Filter-Tips	2 x 50
Elution Tubes (1.5 mL)	2 x 50
Sample Tubes (2.0 mL)	50
Q-Card§	1
Quick-Start Protocol	1

* Kunitz units, defined as the amount of DNase I that causes an increase in A_{260} of 0.001 per minute per milliliter at 25°C, pH 5.0, with highly polymerized DNA as the substrate (1).

† Contains chaotropic salt. Not compatible with disinfecting agents containing bleach; see page 6 for Safety Information.

‡ Contains sodium azide as a preservative.

§ The information encoded in the bar code on the Q-Card is needed for reagent data tracking using the EZ2 Connect instrument.

Shipping and Storage

The EZ2 AllPrep DNA/RNA FFPE Kit is shipped at room temperature (15–25°C). Upon receipt, store the DNase I at 2–8°C. Store all other kit components dry at room temperature.

When stored properly, (buffers and) reagent cartridges are stable until the expiration date on the Q-Card and the kit label.

The EZ2 AllPrep DNA/RNA FFPE Kit contains a ready-to-use Proteinase K solution, which is supplied in a specially formulated storage buffer. Proteinase K is stable for at least 1 year after delivery when stored at room temperature. If ambient temperatures exceed 25°C, we suggest storing Proteinase K at 2–8°C.

Intended Use

The EZ2 AllPrep DNA/RNA FFPE Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the product. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

The EZ2 AllPrep DNA/RNA FFPE Kit is intended to be used on EZ2 Connect instruments, including EZ2 Connect, EZ2 Connect Fx, and EZ2 Connect MDx.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety**, where you can find, view, and print the SDS for each QIAGEN kit and kit component.

CAUTION 	DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Buffers in the EZ2 AllPrep DNA/RNA FFPE cartridge contain chaotropic salts, which can form highly reactive compounds when combined with bleach. If liquid containing these buffers is spilt, clean with a suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

If liquid containing potentially infectious agents is spilt on the EZ2 Connect, please refer to the instrument user manual for decontamination instructions.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, the components of the EZ2 AllPrep DNA/RNA FFPE Kit are tested against predetermined specifications to ensure consistent product quality.

Introduction

The EZ2 AllPrep DNA/RNA FFPE Kit is specially designed for automated simultaneous purification of genomic DNA and total RNA from formalin-fixed, paraffin-embedded (FFPE) tissue sections. DNA and RNA are released sequentially by differential solubilization of the same precious FFPE sample. After solubilization, both nucleic acids are treated separately to remove formaldehyde cross-links and then purified. In contrast to other procedures where either the biological sample or the purified total nucleic acids are divided into two before being processed separately, with the AllPrep FFPE procedure, pure DNA and RNA are obtained from the entire sample.

Due to fixation and embedding conditions, nucleic acids in FFPE samples are usually heavily fragmented and chemically modified by formaldehyde. Therefore, nucleic acids isolated from FFPE samples are often of a lower molecular weight than those obtained from fresh or frozen samples. The degree of fragmentation depends on the type and age of the sample, and on the conditions for fixation, embedding, and storage of the sample. Although formaldehyde modification cannot be detected in standard quality control assays, such as gel electrophoresis or lab-on-a-chip analysis, it does strongly interfere with enzymatic analyses.

While the EZ2 AllPrep DNA/RNA FFPE Kit is optimized to reverse as much formaldehyde modification as possible without further DNA and RNA degradation, nucleic acids purified from FFPE samples should not be used in downstream applications that require high-molecular–weight DNA or full-length RNA. Some applications may require modifications to allow the use of fragmented nucleic acids (e.g., designing small amplicons for PCR and RT-PCR). For cDNA synthesis, gene-specific primers should be used instead of oligo-dT primers. If it is not possible to use gene-specific primers, random primers should be used.

Principle and procedure

This protocol describes usage of the EZ2 AllPrep DNA/RNA FFPE Kit on the EZ2 Connect instrument. For more information or updates, please visit **www.qiagen.com/EZ2Connect-updates**

The EZ2 AllPrep DNA/RNA FFPE procedure integrates well-established magnetic bead-based nucleic acid purification technologies for selective isolation of DNA and RNA from a single FFPE sample into separate eluates. Specially optimized lysis conditions allow the differential release of DNA and RNA from the same FFPE sample and avoid the need for overnight Proteinase K incubation.

Paraffin from FFPE tissue sections is first removed using a non-hazardous paraffine removal solution. The sections are then incubated in an optimized lysis buffer that contains Proteinase K. Under these conditions, RNA is released into solution, whereas genomic DNA and other insoluble material are precipitated. The sample is then centrifuged to yield an RNA-containing supernatant and a DNA-containing pellet, which then undergo separate purification procedures.

The RNA-containing supernatant is incubated at 80°C to reverse formalin crosslinking. This incubation step helps to improve RNA yield and quality, as well as RNA performance in downstream enzymatic assays. The RNA is treated with DNase to digest contaminating genomic DNA and is then bound to magnetic particles on the EZ2 Connect. Contaminants that may interfere with subsequent enzymatic reactions are removed in different washing steps.

The further Proteinase K lysis and crosslink removal of the DNA-containing pellet are conducted on the EZ2 Connect instrument, followed by binding to magnetic particles and optimized washing steps.

Both RNA and DNA are eluted in separate elution tubes in RNase-free water and are ready to use for downstream applications such as RT-PCR, digital PCR, and NGS workflows. Alternatively, they can be stored at –30°C to –15°C.

Automation

This protocol describes the workflow when using the EZ2 Connect instrument. Separation of RNA supernatant and DNA pellet as well as crosslink removal and DNase I digestion of the RNA are carried out manually. RNA binding, washing, elution, and DNA Proteinase K lysis, de-crosslinking, binding, washing, and elution steps are conducted by the EZ2 Connect instrument. For more information about the EZ2 Connect instrument, please visit **www.qiagen.com/EZ2Connect-updates**

Starting material

Typical formalin-fixation and paraffin-embedding procedures result in significant fragmentation of nucleic acids. To limit the extent of nucleic acid fragmentation, please use following guidelines:

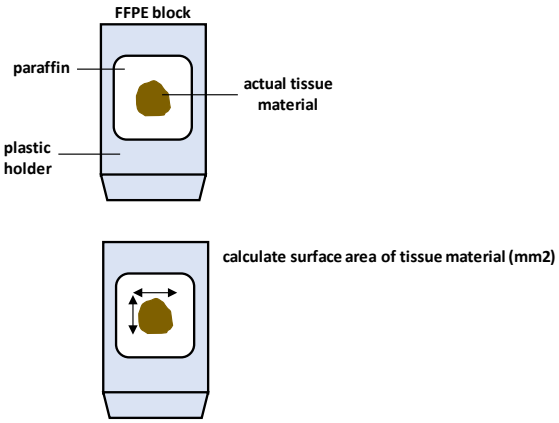
- Fix tissue samples in 4%–10% formalin as quickly as possible after specimen collection.
- Keep formalin fixation time to minimum (longer fixation times lead to more severe DNA/RNA fragmentation, resulting in poor performance in downstream assays).
- Thoroughly dehydrate samples after fixation. This will also remove residual formalin that can inhibit Proteinase K digestion.
- Use low-melting paraffin for embedding, as high temperatures during embedding can cause nucleic acid fragmentation
- Store FFPE samples at low temperatures (2–8°C); storage at room temperature (15–25°C) can lead to nucleic acid degradation

The starting material for nucleic acid purification should be freshly cut sections of FFPE tissue, each with a thickness of 5–10 µm. Thicker sections may result in lower nucleic acid yields, even after prolonged incubation with Proteinase K. Thinner sections can be used but are more difficult to pellet.

Avoid using too much starting material, as this affects lysis efficiency and purification, and can lead to reduced yields and nucleic acid fragmentation.

The amount of starting material specified for use with the EZ2 AllPrep DNA/RNA FFPE Kit refers to the actual tissue material of the FFPE sample, excluding the area of paraffin. The starting material is calculated from the surface area of the tissue, the number of sections, and the thickness of sections. With the EZ2 AllPrep DNA/RNA FFPE Kit, FFPE tissue sections of 5–10 µm thickness can be processed, totaling up to 2 mm^3 of tissue. If there is no information about the nature of your starting material or if the surface area of the sample is high, we recommend starting with one 5–10 µm thick section per preparation.

Sample volume and calculation



Surface area	No. of sections	Total volume
50 mm^2	1 section of 10 µm thickness	0.5 mm^3
	2 sections of 10 µm thickness	1 mm^3
	4 sections of 10 µm thickness	2 mm^3
100 mm^2	1 section of 10 µm thickness	1 mm^3
	2 sections of 10 µm thickness	2 mm^3
200 mm^2	1 section of 10 µm thickness	2 mm^3

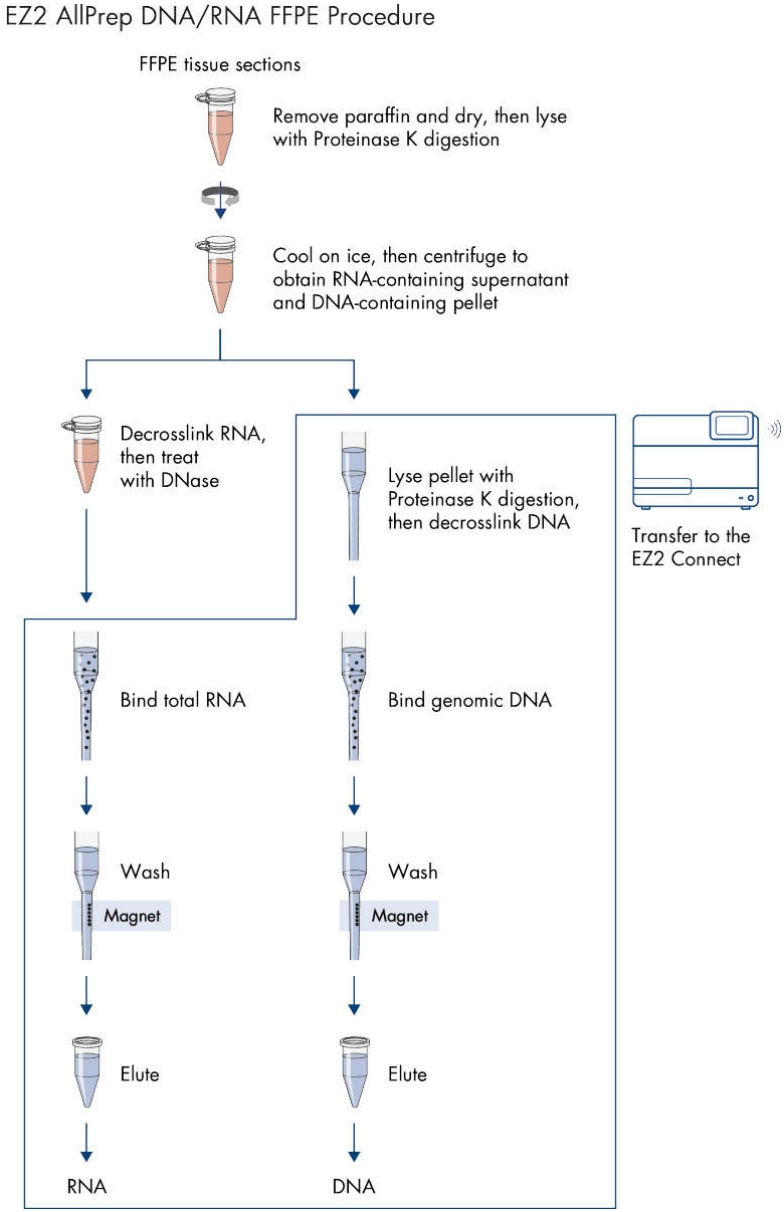


Figure 1. EZ2 AllPrep DNA/RNA FFPE workflow

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- EZ2 Connect instrument (for more information about the EZ2 Connect instrument, please visit **www.qiagen.com/EZ2Connect-updates**)
- Shaker for microcentrifuge tubes capable of incubation at 80°C, for example, the ThermoMixer® Comfort (cat. no. 5355 000.011) with appropriate block from Eppendorf® (**www.eppendorf.com**)
- Microcentrifuge with rotor for 2 mL tubes (up to 21,000 x *g*)
- Pipettors (2–1000 µL)
- Microcentrifuge Tubes (e.g., Safe-Lock® Tubes [Eppendorf, cat. no. 0030 120.086 or 0030 120.094] or SafeSeal microcentrifuge tubes [Sarstedt®, cat no. 72.706 or 72.695.500])

Important Notes

Deparaffinization using Paraffin Removal Solution

Prior to nucleic acid purification, the paraffin in an FFPE sample needs to be removed to allow exposure of the sample to Proteinase K. Paraffin Removal Solution dissolves paraffin efficiently and allows deparaffinization without further washing steps. The volume of Paraffin Removal Solution needed for deparaffinization depends on the amount of sample material. One set of Paraffin Removal Solution is sufficient for deparaffinization of 50 samples consisting of up to two 10 µm sections or one 20 µm section. For larger samples, the volume of Paraffin Removal Solution required is doubled.

Copurification of nucleic acids and DNA/RNA quality and yield

FFPE tissue material presents challenges not only for the DNA/RNA extraction method itself but also for the determination of DNA/RNA quality and quantity. Generally, DNA/RNA yield from FFPE samples varies greatly, depending on the tissue type, as well as fixation and embedding conditions.

Separation of RNA from DNA in the AllPrep protocol is highly effective. This may result in lower A_{260} values when measuring the concentration of DNA purified using the EZ2 AllPrep DNA/RNA FFPE Kit compared to other methods. As A_{260} values measure both DNA and RNA, lower A_{260} values from DNA purified using the EZ2 AllPrep DNA/RNA FFPE Kit may indicate high DNA purity and the absence of contaminating RNA. Higher A_{260} values from DNA purified using alternative methods may indicate the presence of significant amounts of contaminating RNA.

Especially for DNA from samples with heavy fragmentation, fluorometric methodologies using dyes specific for dsDNA (e.g., Qubit®) might show significantly lower DNA recovery as compared to A_{260} absorbance. In addition, yield and PCR performance do not necessarily

correlate; high yields of DNA or RNA as determined by either of the abovementioned methods might not show good PCR performance. This could be due to the quality of the FFPE sample with regard to DNA/RNA fragmentation status and/or the efficiency of crosslink reversal prior to DNA/RNA extraction. DNA/RNA of a more fragmented status shows far better PCR performance for short amplicons in PCR (<100 bp) than DNA/RNA of higher molecular weight. However, highly fragmented DNA/RNA will not be suitable for PCR applications with amplicons larger than the size of the extracted DNA/RNA fragments. If de-crosslinking during DNA/RNA purification is insufficient, the extracted DNA/RNA will not be properly accessible despite sufficient integrity and poses a poor template for amplification of both small and large fragments in PCR. Thus, DNA/RNA yield measured by PCR may differ between large amplicon and short amplicon PCR systems and might also deviate from values obtained by UV-Vis–based or fluorometric quantification technologies.

It is recommended to use more than one quality control measure to evaluate DNA/RNA quality and quantity, focusing on which downstream application the DNA/RNA is intended to be used in. The EZ2 AllPrep DNA/RNA FFPE Kit provides an optimized workflow for extraction of DNA and RNA for use in PCR, digital PCR, and NGS analysis.

RNA purified using the EZ2 AllPrep DNA/RNA FFPE Kit is virtually free of DNA contamination, as the RNA purification procedure includes a DNase digestion step.

Preparation of buffers

Preparing DNase I stock solution

Prepare DNase I stock solution by dissolving the lyophilized DNase I (1500 Kunitz units) in 550 µL RNase-free water. In some cases, the vial of DNase may appear to be empty. This is due to lyophilized enzyme sticking to the septum. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. Do not vortex.

Insoluble material may remain when dissolving DNase. This does not affect DNase performance. Due to the production process, insoluble material may be present in the lyophilized DNase. However, rigorous QC tests are carried out to ensure that DNase activity remains consistent from lot to lot.

> **Note**: Do not vortex reconstituted DNase I. DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the vial.

For long-term storage of DNase I, remove the stock solution from the vial, divide it into single-use aliquots, and store at –30°C to –15°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 4 weeks. Do not refreeze the aliquots after thawing.

Preparing Buffer ATL

Before starting the procedure, check whether precipitate has formed in Buffer ATL. If necessary, dissolve by heating to 70°C with gentle agitation.

Working with the EZ2 Connect instruments

The main features of EZ2 Connect instruments include:

- Purification of high-quality nucleic acids from up to 24 samples per run
- Small footprint to save laboratory space
- Preprogrammed protocols for nucleic acid purification
- Prefilled, sealed reagent cartridges for easy, safe, and fast run setup
- Complete automation of nucleic acid purification, from opening of reagent cartridges to elution of nucleic acids, with no manual centrifugation steps
- Optional bar code reading and sample tracking
- Kit data tracking with the Q-Card provided in the kit
- UV LED to help eliminate sample carryover from run-to-run and to allow pathogen decontamination on the worktable surfaces

  Note: UV decontamination helps to reduce possible pathogen contamination of the EZ2 Connect. The efficiency of inactivation has to be determined for each specific organism and depends, for example, on layer thickness and sample type. QIAGEN cannot guarantee complete eradication of specific pathogens.

EZ2 Connect Reagent cartridges

Reagents for the purification of nucleic acids from a single sample are contained in a single reagent cartridge (Figure 2). Each well of the cartridge contains a particular reagent, such as magnetic particles, lysis buffer, wash buffer, or elution buffer. Positions 11 and 12 can be equipped individually. Details on preparation of these positions are displayed during the run setup on the LED display of the EZ2 Connect.

Figure 2. Ease of worktable setup using reagent cartridges. (A) A sealed, prefilled reagent cartridge. Fill levels vary, depending on the type of reagent cartridge. **(B)** Loading reagent cartridges into the cartridge rack. The cartridge rack itself is labeled with an arrow to indicate the direction in which reagent cartridges must be loaded. **(C)** Schematic representation of the reagent cartridge with positions 1–12 marked.

EZ2 Connect tip racks

The EZ2 Connect tip racks hold tips inserted into tip holders and tubes for samples or elution (Figure 3). Details on how to equip the tip racks are displayed during the run setup on the LED display of the EZ2 Connect. The display also shows protocol status during the automated purification procedure.

Figure 3. EZ2 Connect tip racks, tip holders, and filter-tips.

Worktable

The worktable of EZ2 Connect instruments is where the user loads cartridge and tip racks (Figure 4).

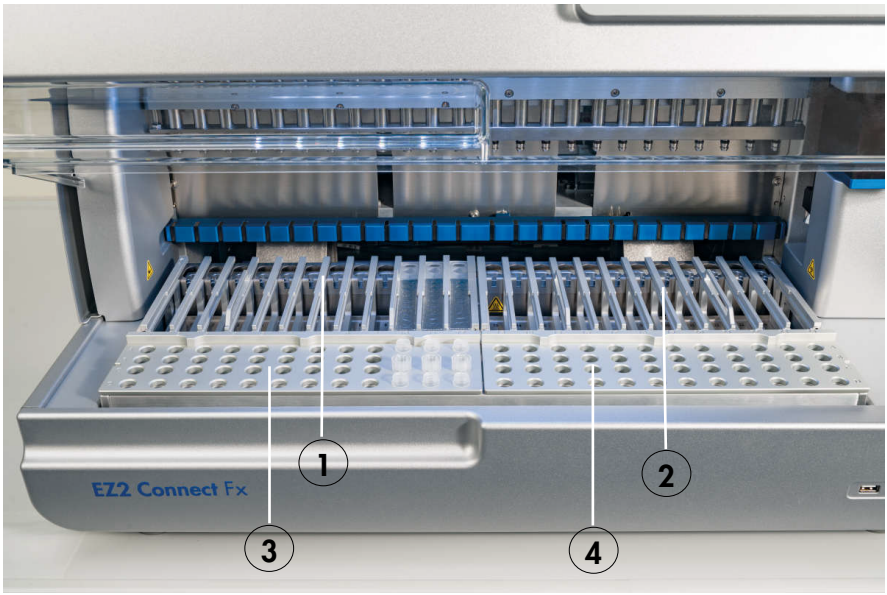


Figure 4. EZ2 Connect Worktable.

1. EZ2 Connect Cartridge Rack – left
2. EZ2 Connect Cartridge Rack – right
3. EZ2 Connect Tip Rack – left
4. EZ2 Connect Tip Rack – right

Operation of the EZ2 Connect

The EZ2 Connect provides various features to support the sample preparation workflow. These include functions for remote access via QIAsphere®, data input via bar code reading, data storage and transfer, report generation, and guided instrument maintenance. For more information about these features, please refer to the *EZ2 Connect and EZ2 Connect Fx User Manual*.

Workflow of the EZ2 Connect AllPrep DNA/RNA FFPE operation

Prepare samples to obtain RNA lysate and DNA pellet.

↓

Switch on the EZ2 instrument

↓

Follow onscreen messages for worktable setup

↓

Start the protocol

↓

Collect purified nucleic acids

Protocol: EZ2 AllPrep DNA/RNA FFPE Kit

Important notes before starting

- If any run has been performed before starting the EZ2 AllPrep DNA/RNA FFPE protocol, run the "Cooling" protocol from the home screen first, in order to ensure that the heating block is at room temperature to protect your RNA and DNA from potential degradation.
- Before first use, resuspend the DNase I with 550 µL of the supplied RNase-free water. The solution can be stored at 2–8°C for up to 4 weeks or should be aliquoted and stored at –30°C to –15°C for extended time periods while avoiding freeze-thaw cycles.
- Set a thermal mixer to 56°C.
- Before loading reagent cartridges into the EZ2 Connect instrument, invert them 3 times to mix the magnetic particles and then tap to deposit the reagents at the bottom of the wells. Make sure that the magnetic particles are completely resuspended.

Procedure

1. Trim excess paraffin off the sample block using a scalpel.
2. Cut the paraffin-embedded sample into sections of 10 µm thickness. If the sample surface has been exposed to air, discard the first 2–3 sections. The sample volume should not exceed 2 mm^3.
3. Immediately place the sections into a 2 mL sample tube (provided).

   Optional: Sections can be stored at –30°C to –15°C for 4 weeks. Before proceeding with step 4, thaw samples at room temperature in that case.
4. Add 320 µL Paraffin Removal Solution (PRS) and close the cap.
5. Vortex vigorously for 10 s, and centrifuge briefly to bring the sample to the bottom of the tube.
6. Incubate at 56°C for 3 min and centrifuge at 20,000 x *g* for 2 min.
7. Carefully remove the supernatant by pipetting without disturbing the pellet.

8. Resuspend the pellet by adding 150 µl Buffer PKD and flick the tube to loosen the pellet. Add 10 µL Proteinase K and mix by vortexing briefly.
9. Place the tubes in a thermal mixer and incubate at 56°C for 15 min at 500 rpm. Incubate on ice for 3 min. Set a thermal mixer to 80°C.
10. Centrifuge for 15 min at 20,000 x *g*. Carefully transfer the supernatant, without disturbing the pellet, to a new 1.5 mL microcentrifuge tube for RNA preparation (step 12).
11. Keep the pellet for DNA preparation (step 16).

    Optional: Both RNA lysate (step 10) and DNA pellet (step 11) can be stored at –30°C to –15°C for up to 1 week. Before proceeding with steps 12 and 16, thaw samples at room temperature in that case. We recommend to briefly vortex and spin down the sample before proceeding.

RNA preparation

12. Incubate the RNA-containing supernatant from step 10 at 80°C for 15 min. Incubate on ice for 3 min.

    Note: RNA decrosslinking can be extended up to 60 min at 80°C for better PCR performance. However, this can negatively impact other downstream applications such as next-generation sequencing.
13. Add 16 µL DNase booster buffer and mix by vortexing.
14. Add 10 µL DNase I; mix gently by inverting the tube and spin down.
15. Incubate at room temperature for 15 min.

DNA preparation

16. Resuspend the DNA-containing pellet from step 11 in 180 µL Buffer ATL, add 40 µL Proteinase K, and mix by vortexing briefly. Spin down to collect the sample at the bottom of the tube.
17. Overlay this suspension carefully with 200 µL PRS.

EZ2 preparation

18. Turn on the EZ2 Connect instrument.
19. Tap **RNA** on the Applications panel, select the **AllPrep DNA/RNA FFPE Kit**, and press **Next**. Follow onscreen instructions for selection of protocol, parameter definition, sample position selection, sample IDs, and worktable setup.

    Important: Ensure the heating block of the EZ2 instrument is at room temperature.

    Note: The choice of the "Standard" or "Fast" protocol version can influence the DNA quality. While "Standard" is recommended in most cases, the "Fast" protocol version may be beneficial for downstream applications relying on long and intact DNA fragments such as next-generation sequencing.
20. Load the EZ2 AllPrep DNA/RNA FFPE reagent cartridges into the EZ2 Connect Cartridge Rack.
21. Transfer the RNA lysate from step 15 into position 12 of the EZ2 AllPrep DNA/RNA FFPE cartridge (see Figure 2C).
22. Place the 2 mL sample tube containing the DNA pellet from step 17 into position 11 of the reagent cartridge (see Figure 2C).
23. Open instrument hood. Load the EZ2 Connect Cartridge Rack into the EZ2 Connect instrument.
24. Remove caps of all tubes and prepare the EZ2 Connect Tip Rack as follows:
    - Position A: Tip holder with Filter Tip
    - Position B: Tip holder with Filter Tip
    - Position C: new 1.5 mL elution tube
    - Position D: new 1.5 mL elution tube
25. Place the EZ2 Connect Tip Rack into the EZ2 Connect instrument and start the protocol.
26. The display will show "Protocol finished" when the run is completed. Select **Finish**.

27. Open the instrument hood. Remove the elution tube containing the purified DNA from position C and the elution tube containing the purified RNA from position D of the EZ2 Connect Tip Rack. Discard the used EZ2 cartridge including the liquid waste.

    Optional: Follow onscreen instructions for UV decontamination of worktable surfaces.

    Perform regular maintenance after each run. Press **Finish** to return to the home screen.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page in our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information or protocols in this handbook (for contact information, visit **support.qiagen.com**).

	Comments and suggestions
Poor nucleic acid yield or quality	
a) Poor quality of starting material	Samples that were fixed for over 20 hours or stored for very long periods of time may contain very little usable nucleic acids. Sections that were mounted on microscope slides may yield very little usable nucleic acids due to prolonged exposure to air.
b) Insufficient reagent aspirated	After inverting the reagent cartridges to resuspend the magnetic particles, make sure to tap the cartridges to deposit the reagents at the bottom of the wells.
c) Magnetic particles not completely resuspended	Make sure to resuspend the magnetic particles thoroughly before loading the reagent cartridges into the holder.
d) Low RNA/DNA yield	Make sure that the heating block is at room temperature before starting your run to protect your RNA and DNA from potential degradation. This can be done by starting the "Cooling" protocol from the EZ2 Connect main screen.
e) Low DNA yield	Make sure that the resuspended DNA pellet was overlaid with Paraffin Removal Solution before loading the sample tube into the cartridge (protocol steps 16 and 17).
f) Sub-optimal PCR performance of RNA	Extend the decrosslinking time of the RNA lysate to up to 60 min at 80°C for optimized PCR performance. However, this can negatively impact its integrity.
g) Sub-optimal PCR performance of DNA	Make sure to use the "Standard" version of the EZ2 AllPrep DNA/RNA FFPE protocol as this provides optimized DNA decrosslinking. However, this can negatively impact its integrity.
h) Poor RNA integrity (on Bioanalyzer/QIAxcel)	Make sure to decrosslink the RNA lysate only for 15 min at 80°C as extended times can lead to further fragmentation of your sample.

		Comments and suggestions
i)	Poor DNA integrity on TapeStation/Agarose gel	Make sure to use the "Fast" version of the EZ2 AllPrep DNA/RNA FFPE protocol as this provides less fragmented DNA. However, this can negatively impact its performance in other downstream applications such as qPCR.

General handling

a)	Error message in instrument display	Refer to the user manual supplied with your EZ2 Connect instrument.
b)	Report file not printed	Check whether the printer is connected to the EZ2 Connect via the "PC/Printer" serial port. Check whether the serial port is set for use with a printer.
c)	Paraffin top layer solidifies (partially) upon cooling (step 7)	Increase the volume of PRS and repeat the heating step 6 or use fewer sections.

References

1. Kunitz, M. (1950). Crystalline desoxyribonuclease; isolation and general properties; spectrophotometric method for the measurement of desoxyribonuclease activity. J. Gen. Physiol. **33**, 349–363.
2. Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997). Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. BioTechniques. **22**, 474.

Appendix A: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. To create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice when aliquots are pipetted for downstream applications.

To remove RNase contamination from bench surfaces, nondisposable plasticware, and laboratory equipment (e.g., pipettes and electrophoresis tanks), general laboratory reagents can be used. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 31), or rinse with chloroform* if the plasticware is chloroform-resistant.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

To decontaminate electrophoresis tanks, clean with detergent (e.g., 0.5% SDS)*, rinse with RNase-free water, rinse with ethanol (if the tanks are ethanol-resistant), and allow to dry.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent,* thoroughly rinsed and oven baked at 240°C for at least 4 hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate), as described in "Solutions" below.

Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 mL DEPC to 100 mL of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

presence of Tris buffers and decomposes rapidly into ethanol* and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer.

Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues has been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

Note: AllPrep buffers are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

* Plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier's instructions.

Appendix B: Storage, Quantification and Determination of Quality of RNA

Storage of RNA

Purified RNA may be stored at –30°C to –15°C or –90°C to –65°C in RNase-free water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

The concentration of RNA can be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (see "Spectrophotometric quantification of RNA" below). For small amounts of RNA, however, it may not be possible to accurately determine amounts photometrically. Small amounts of RNA can be quantified using the QIAxcel® Advanced system (**qiagen.com/QIAxcel**) or Agilent® 2100 Bioanalyzer, quantitative RT-PCR, or fluorometric quantification.

Spectrophotometric quantification of RNA

To ensure significance, A_{260} readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per mL (A_{260}=1 → 44 µg/mL). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH.* As discussed below (see "Purity of RNA", page 34), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry.

This can be accomplished by washing cuvettes with 0.1 M NaOH, 1 mM EDTA,* followed by washing with RNase-free water (see "Solutions", page 31).

Use the buffer in which the RNA is diluted to zero the spectrophotometer. An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 µL
Dilution = 10 µL of RNA sample + 490 µL of 10 mM Tris·Cl,* pH 7.0 (1/50 dilution)
Measure absorbance of diluted sample in a 1 mL cuvette (RNase-free)
A_{260} = 0.2

Concentration of RNA sample	= 44 µg/mL x A_{260} x dilution factor
	= 44 µg/mL x 0.2 x 50
	= 440 µg/mL
Total amount	= concentration x volume in milliliters
	= 440 µg/mL x 0.1 mL
	= 44 µg of RNA

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination (2).

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier

For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1* in 10 mM Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution used for dilution.

For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 44 µg/mL RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Spectrophotometric quantification of RNA", page 33).

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. While the EZ2 AllPrep DNA/RNA FFPE procedure will remove the vast majority of cellular DNA, trace amounts may still remain, depending on the amount and nature of the sample.

For analysis of very low abundance targets, any interference by residual DNA contamination can be detected by performing real-time RT-PCR control experiments in which no reverse transcriptase is added prior to the PCR step.

To prevent any interference by DNA in real-time RT-PCR applications, such as with Applied Biosystems® and Rotor-Gene® instruments, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified. QuantiTect Primer Assays from QIAGEN are designed for SYBR® Green-based real-time RT-PCR analysis of RNA sequences (without detection of genomic DNA) where possible (see **www.qiagen.com/GeneGlobe**). For real-time RT-PCR assays where amplification of genomic DNA cannot be avoided, the QuantiTect Reverse Transcription Kit provides fast cDNA synthesis with integrated removal of genomic DNA contamination (cat. no. 205311).

* Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.

Integrity of RNA

The integrity and size distribution of total RNA purified with the EZ2 AllPrep DNA/RNA FFPE Kit can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining* or by using the QIAxcel Advanced system or Agilent 2100 Bioanalyzer. For intact RNA, the respective ribosomal RNAs should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S rRNA should be approximately 2:1. However, due to effects of formalin fixation, embedding, and storage, RNA from FFPE specimens will usually show varying degrees of degradation. In some cases, there may be no discernible rRNA peaks left.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Appendix C: Storage, Quantification, and Determination of Quality of Genomic DNA

Storage of DNA

For long-term storage, purified DNA can be stored at to –30°C to –15°C. Avoid any contamination, as this may lead to DNA degradation. We recommend storing samples in aliquots to avoid repeated freezing and thawing.

Quantification of DNA

The concentration of DNA can be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (see "Spectrophotometric quantification of DNA" below). For small amounts of DNA, however, it may not be possible to accurately determine amounts photometrically. Small amounts of DNA can be quantified using quantitative real-time PCR, or fluorometric quantification.

Spectrophotometric quantification of DNA

DNA concentration can be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer using a quartz cuvette. For greatest accuracy, readings should be between 0.1 and 1.0. Using a standard 1 cm path length, an absorbance of 1 unit at 260 nm corresponds to 50 µg genomic DNA per mL (A_{260} =1 → 50 µg/mL). This relation is valid only for measurements made at neutral pH. Therefore, samples should be diluted in a low-salt buffer with neutral pH (e.g., Tris·Cl, pH 7.0)*. Use the buffer in which the DNA is diluted to zero the spectrophotometer. An example of the calculation involved in DNA quantification is shown as follows:

* When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Volume of DNA sample = 100 µL
Dilution = 20 µL of DNA sample + 180 µL of buffer (1/10 dilution)
Measure absorbance of diluted sample in a 0.2 mL cuvette
A_{260} = 0.2

Concentration of DNA sample	= 50 µg/mL x A_{260} x dilution factor
	= 50 µg/mL x 0.2 x 10
	= 100 µg/mL
Total amount	= concentration x volume of sample in milliliters
	= 100 µg/mL x 0.1 mL
	= 10 µg of DNA

RNA concentration can also be determined by measuring the absorbance at 260 nm. If the eluate contains both DNA and RNA, a fluorometer must be used to quantify the DNA.

Determination of DNA purity

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of DNA purity with respect to contaminants that absorb UV light, such as protein. The A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination (2). For accurate A_{260}/A_{280} values, we recommend measuring absorbance in a slightly alkaline buffer (e.g., 10 mM Tris·Cl, pH 7.5). Make sure to zero the spectrophotometer with the appropriate buffer.

Pure DNA has an A_{260}/A_{280} ratio of 1.7–1.9. Scanning the absorbance from 220–320 nm will show whether there are contaminants affecting absorbance at 260 nm. Absorbance scans should show a peak at 260 nm and an overall smooth shape.

Determination of DNA length

The precise length of genomic DNA can be determined by pulsed-field gel electrophoresis (PFGE) through an agarose gel. The DNA should be concentrated by alcohol* precipitation and reconstituted by gentle agitation in approximately 30 µL TE buffer, pH 8.0,* for at least 30 minutes at 60°C. Avoid drying the DNA pellet for more than 10 minutes at room temperature (15–25°C), since over-dried genomic DNA is very difficult to redissolve. Load 3–5 µg of DNA per well. Standard PFGE conditions are as follows:

- 1% agarose* gel in 0.5x TBE electrophoresis buffer*
- Switch intervals = 5–40 s
- Run time = 17 h
- Voltage = 170 V

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Appendix D: Supplementary Protocol for FFPE Cores using the EZ2 AllPrep DNA/RNA FFPE Kit

As an alternative to sectioning using a microtome, biopsy needles can be used to obtain core punches from FFPE specimens. The diameter of these cores is typically several orders of magnitude greater than the thickness of microtome sections. This protocol has been specially adapted from the EZ2 AllPrep DNA/RNA FFPE protocol for the simultaneous purification of genomic DNA and RNA from FFPE cores using the EZ2 AllPrep DNA/RNA FFPE Kit.

Starting material

Starting material for nucleic acid purification should be one FFPE core with a maximum diameter of 3 mm and a length of 3 mm after excess paraffin is trimmed off. If there is no information about the nature of your starting material, we recommend starting with no more than 10 mg FFPE core per preparation. Do not use more than 25 mg of FFPE core per preparation.

Procedure

1. Place FFPE core material into a 2 mL sample tube and close the lid.

   Do not use more than the recommended maximum amount of starting material. See section “Starting material”.

2. Disrupt and homogenize the FFPE core using the TissueLyser III.

   Place a stainless steel bead (5 mm diameter) in the 2 mL sample tube containing the sample. Place the tubes in the TissueLyser Adapter Set 2 x 24. Operate the TissueLyser III for 30 s at 20 Hz.

   Complete disruption and homogenization may not always be possible. However, small amounts of debris are usually digested in the Proteinase K step.

   Leave the stainless steel bead in the 2 mL sample tube and proceed to step 3.

3. Add 320 µL Paraffin Removal Solution (PRS) and close the lid.
4. Vortex vigorously for 10 s, and centrifuge briefly to bring the sample to the bottom of the tube.
5. Incubate at 56°C for 3 min and centrifuge at 20,000 x *g* for 2 min.
6. Remove the stainless steel bead and the supernatant without disturbing the pellet.

   For bead removal, a magnet can be slid along the outside of the reaction tube, so that the bead is indirectly moved to the rim of the tube. Subsequently remove the supernatant by pipetting. Carefully remove any residual liquid using a fine pipet tip.

   Note: In some cases the pellet may be loose. Remove the stainless steel bead and supernatant carefully.

7. Resuspend the pellet by adding 150 µL Buffer PKD and flicking the tube to loosen the pellet. Add 10 µL Proteinase K and mix by vortexing.
8. Place the tubes in a thermal mixer and incubate at 56°C for 15 min at 500 rpm.

   Depending on the sample material, the sample may not be completely lysed. This does not affect the procedure. Proceed to step 9.

9. Incubate on ice for 3 min.

Complete cooling is important for efficient precipitation in step 10. Set a thermal mixer to 80°C.

10. Centrifuge for 15 min at 20,000 x *g*.
11. Carefully transfer the supernatant without disturbing the pellet to a new 1.5 mL microcentrifuge tube for RNA preparation. Keep the pellet for DNA preparation.

    Note: Depending on the amount and nature of the FFPE sample, the pellet may be very small or difficult to see. If the pellet is aspirated with the supernatant, allow the pellet to drop slowly to the bottom of the tube and use the pipette tip to reattach the pellet to the tube. Alternatively, centrifuge the supernatant again.

    Optional: Both RNA lysate and DNA pellet can be stored at –30°C to –15°C for up to 1 week. Before proceeding, thaw samples at room temperature in that case. We recommend to briefly vortex and spin down the sample before proceeding.
12. Proceed to step 12 of the EZ2 AllPrep DNA/RNA FFPE protocol for RNA purification or to step 16 of the EZ2 AllPrep DNA/RNA FFPE protocol for DNA purification.

Ordering Information

Product	Contents	Cat. no.
EZ2 AllPrep DNA/RNA FFPE Kit (48)	For 48 preps: Paraffin Removal Solution, EZ2 AllPrep DNA/RNA FFPE cartridge, Filter Tips and Holders, Tubes, Proteinase K, DNase I, RNase-free Water, and Buffers	954734
EZ2 Connect	Benchtop instrument for automated isolation of nucleic acids from up to 24 samples in parallel, using sealed prefilled cartridges; includes 1-year warranty on parts and labor.	9003210
Accessories and Reagents		
QIAGEN Proteinase K (2 mL)	2 mL (>600 mAU/mL, solution)	19131
Filter Tips and Holders, EZ1&2 (50)	50 Disposable Filter-Tips, 50 Disposable Tip Holders; additional tips and holders for use with EZ1&2 Kits	994900
TissueLyser III	Bead mill, 100–120/220–240 V, 50/60 Hz; requires TissueLyser Adapters (available separately)	9003240
TissueLyser Adapter Set 2 x 24	Two sets of adapter plates and 2 racks for use with 2 mL microcentrifuge tubes on the TissueLyser II & III	69982
Stainless Steel Beads, 5 mm (200)	200 stainless steel beads (5 mm diameter), suitable for use with TissueLyser systems	69989
QIAxcel Advanced system	Robotic workstation for automated capillary electrophoresis, notebook	9002123

Product	Contents	Cat. no.
	computer, and QIAxcel software; includes installation, application training and one-year warranty on labor, shipping and parts.	
Rotor-Gene Q 2plex Platform	Real-time PCR cycler with 2 channels (green, yellow), laptop computer, software, accessories; includes 1-year warranty on parts and labor; installation and training not included	9001550
QuantiTect Reverse Transcription Kit (50)	For 50 x 20 µL reactions: 100 µL 7x gDNA Wipeout Buffer, 50 µL Quantiscript Reverse Transcriptase, 200 µL 5x Quantiscript RT Buffer, 50 µL RT Primer Mix, 1.9 mL RNase-Free Water	205311

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Date	Description
February 2022	Initial release
July 2023	Revised “Starting material” section under Introduction. Edited “Important Notes” section. Updated “Protocol: EZ2 AllPrep DNA/RNA FFPE Kit”. Added “Appendix D: Supplementary Protocol for FFPE Cores using the EZ2 AllPrep DNA/RNA FFPE Kit”. Updated Ordering Information.

Notes

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