March 2022

EZ2® RNA FFPE Handbook

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

Sample to Insight

QIAGEN

Contents

Kit Contents

EZ2 RNA FFPE Kit **Catalog no.** **No. of reactions**	**(48)** **959734** **48**
Paraffin Removal Solution	20 ml
RNase-Free DNase I (lyophylized)	1500 units*
DNase Booster Buffer	2 ml
RNase-Free Water (for use with RNase-free DNase I)	1.9 ml
EZ2 RNA FFPE Cartridges†	48
Disposable Tip Holders	2 x 50
Disposable Filter-Tips	2 x 50
Tubes 1.5 ml	2 x 50
Tubes 2 ml	1 x 50
Q-Card‡	1
Quick-Start Protocol	1

* Kunitz units, defined as the amount of DNase I that causes an increase in A260 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 5 for Safety Information.

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

Shipping and Storage

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

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

Intended Use

The EZ2 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 products. 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 RNA FFPE Kit is intended to be used with 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 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 instrument, 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 RNA FFPE Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Formalin fixation followed by paraffin embedding of tissue specimens is a standard method for preserving histological structures within tissues. Exposure to formalin results in cross-linkage and thereby stabilization of proteins and nucleic acids. In addition to studying morphology, the resulting formalin-fixed, paraffin-embedded (FFPE) tissue samples are valuable for molecular analyses as well. However, RNA preparation from FFPE tissue is associated with several challenges. Yields are often low due to the limited availability of input material and the compromised quality of RNA due to fixation and long-term storage of samples. RNA isolated from FFPE samples is often of a lower molecular weight than those obtained from fresh or frozen samples. The degree of this fragmentation depends on the type and age of the sample and on the conditions for fixation, embedding, and storage of the sample. Frequently, applications for molecular analyses may require modifications to allow the use of fragmented RNA (e.g., designing small amplicons for RT-PCR). For cDNA synthesis, either random or gene-specific primers should be used instead of oligo-dT primers.

Additionally, recovery of amplifiable RNA strongly depends on removal of formalin-induced crosslinks. 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.

The EZ2 RNA FFPE Kit provides convenient, streamlined procedures for efficient, automated purification of RNA from challenging FFPE tissue sections including dedicated steps for the removal of formalin-induced crosslinks.

Principle and procedure

This protocol describes usage of the EZ2 RNA FFPE Kit on the EZ2 Connect instruments. The EZ2 RNA FFPE procedure removes paraffin with the non-hazardous Paraffin Removal Solution, so without use of hazardous solvents or the need to trim off excess paraffin from the FFPE block

in advance. All steps following the removal of paraffin are carried out on the EZ2 Connect instrument. Proteinase K digestion in an optimized lysis buffer ensures complete lysis of even difficult-to-lyse tissue and releases RNA. Formalin-induced crosslinks are efficiently removed in an incubation step at elevated temperature. This facilitates the recovery of high amounts of amplifiable RNA. Next, RNA is treated with DNase to digest contaminating genomic DNA and is then bound to magnetic particles. Contaminants that may interfere with subsequent enzymatic reactions are removed in three washing steps.

RNA is eluted in RNase-free water. Isolated RNA is compatible with RT-PCR, digital PCR, and NGS workflows. If necessary, RNA can be stored long term at –30 to –15°C.

Automation

This protocol describes the workflow when using the EZ2 Connect instruments. The EZ2 Connect instruments can perform all steps following deparaffinization of the sample in an automated procedure. This includes lysis with Proteinase K, crosslink removal, and DNase digestion. RNA binding, washing, and elution steps are then conducted using magnetic particle technology. Up to 24 samples may be processed in a single run allowing flexibility through a selection of different protocol options depending on intended downstream use of the isolated RNA (see the "Workflow" section, page 12, for details about protocol variants).

Magnetic-particle technology combines the speed and efficiency of silica-based RNA purification with the convenient handling of magnetic particles. RNA is isolated from lysates in one step through its binding to the silica surface of the particles in the presence of a chaotropic salt. The particles are separated from the lysates using a magnet. The RNA is then efficiently washed and eluted.

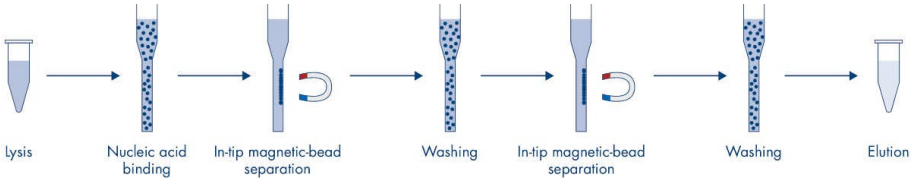


Figure 1. Principle of the EZ2 technology.

Starting material

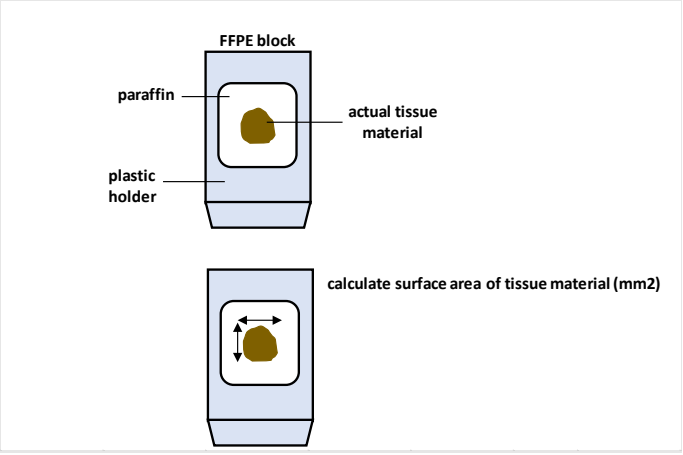
Typical formalin-fixation and paraffin-embedding procedures result in significant fragmentation of nucleic acids. To limit the extent of nucleic acid fragmentation, please use following guidelines:

- 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 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 RNA fragmentation.

Sample material for RNA extraction from FFPE tissue is prepared as 5 to 10 µm sections cut from a FFPE block using a microtome.

The amount of starting material specified for use with the EZ2 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 RNA FFPE Kit, FFPE tissue sections of 5–10 µm thickness can be processed, totaling up to 4 mm^3 of tissue. In cases where calculating the exact amount is impossible, use no more than 2 sections of 5–10 µm thickness.

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
	8 sections of 10 µm thickness	4 mm^3
100 mm^2	1 section of 10 µm thickness	1 mm^3
	2 sections of 10 µm thickness	2 mm^3
	4 sections of 10 µm thickness	4 mm^3
200 mm^2	1 section of 10 µm thickness	2 mm^3
	2 sections of 10 µm thickness	4 mm^3
400 mm^2	1 section of 10 µm thickness	4 mm^3

RNA quality and yield

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

Furthermore, due to the compromised status of the RNA, determination of yield might vary between different quantification methods. Yield may be determined using UV-Vis–based measurements or, fluorometric methodologies using dyes. However, yield determined by either of these methods and RT-PCR performance may not necessarily correlate; high yields of RNA as determined by either of the abovementioned methods might not show good RT-PCR performance. This could be due to the quality of the FFPE sample regarding the RNA fragmentation status and/or the efficiency of crosslink reversal prior to RNA extraction. RNA of a more fragmented status may show better RT-PCR performance for short amplicons in RT-PCR (<100 bp) than RNA of higher molecular weight. However, highly fragmented RNA will not be suitable for RT-PCR applications with amplicons larger than the size of the extracted RNA fragments. If de-crosslinking during RNA purification is insufficient, the extracted RNA will not be properly accessible despite sufficient integrity and poses a poor template for amplification of both small and large fragments in RT-PCR. Thus, RNA yield measured by RT-PCR may differ between large amplicon and short amplicon RT-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 RNA quality and quantity, focusing on which downstream application the RNA is intended to be used in. The EZ2 RNA FFPE Kit provides an optimized workflow for extraction of RNA for use in RT-PCR, digital PCR, and NGS analysis allowing a selection between a standard protocol and a fast protocol version. The fast protocol allows time-efficient extraction of RNA under milder incubation conditions. Depending on the inherent RNA integrity of the sample, RNA for downstream applications requiring a higher level of integrity can be extracted. The standard protocol contains an incubation step designed for most efficient removal of crosslinks especially for downstream applications such as RT-PCR.

Workflow

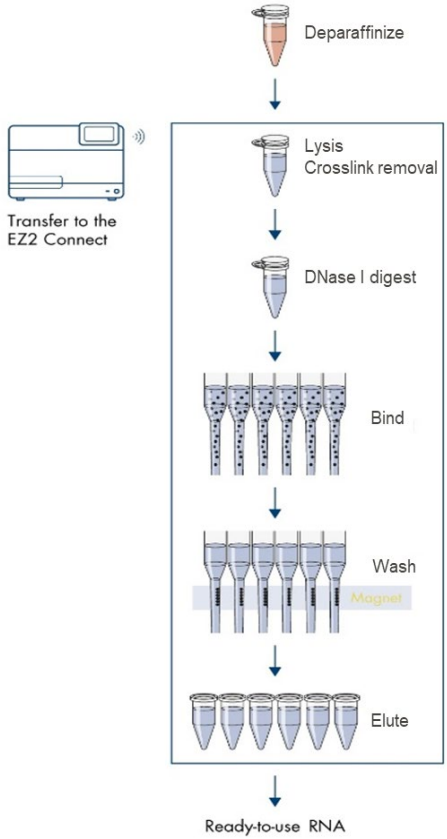


Figure 2. EZ2 RNA FFPE workflow on the EZ2 Connect instrument.

Protocol versions

Two protocol versions for processing the EZ2 RNA FFPE Kit are available: “Standard Protocol” and “Fast Protocol”. Both variants differ from each other with respect to processing parameters like incubation times.

The “Standard Protocol” is optimized to deliver optimal performance in downstream applications that benefit from a higher yield of amplifiable RNA, such as RT-qPCR.

The “Fast Protocol” uses shorter incubation times, which has no impact on performance for most sample types. In addition to the time-saving aspect, the Fast Protocol tends to result in RNA with higher integrity, Steps for upfront manual processing and setup of the work deck do not differ between both variants.

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 (cat. no. 9003210)
- Shaker for microcentrifuge tubes capable of incubation at 56°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)

Important Notes

Starting material

Standard formalin-fixation and paraffin-embedding procedures always result in significant fragmentation and crosslinking of nucleic acids. To limit the extent of nucleic acid fragmentation and crosslinking, be sure to follow recommendations stated above for fixing and embedding conditions.

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, due to insufficient tissue lysis. Avoid using too much starting material, as this also affects lysis efficiency and purification. Therefore, it can lead to reduced yields.

If there is no information about the nature of your starting material, we recommend using no more than 2 5–10 µm thick sections per preparation. If the surface area of the sample is high, we recommend starting with one 5–10 µm thick section per preparation.

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 to –15°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to four weeks. Do not refreeze the aliquots after thawing.

Working with the EZ2 Connect Instrument

The key 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 3). 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 3. 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.

EZ2 Connect tip racks

The EZ2 Connect tip racks hold tips inserted into tip holders and tubes for samples or elution. Details on how to equip the tip racks are displayed during the run setup on the LED display of the EZ2 Connect.

Figure 4. The EZ2 Connect Tip Rack (A) has 4 positions label A–D by engravings. It is designed to hold sample and elution tubes as well as tips in their respective tip holders (B)

Worktable

The worktable of EZ2 Connect instruments is where the user equipped cartridge and tip racks (Figure 5).

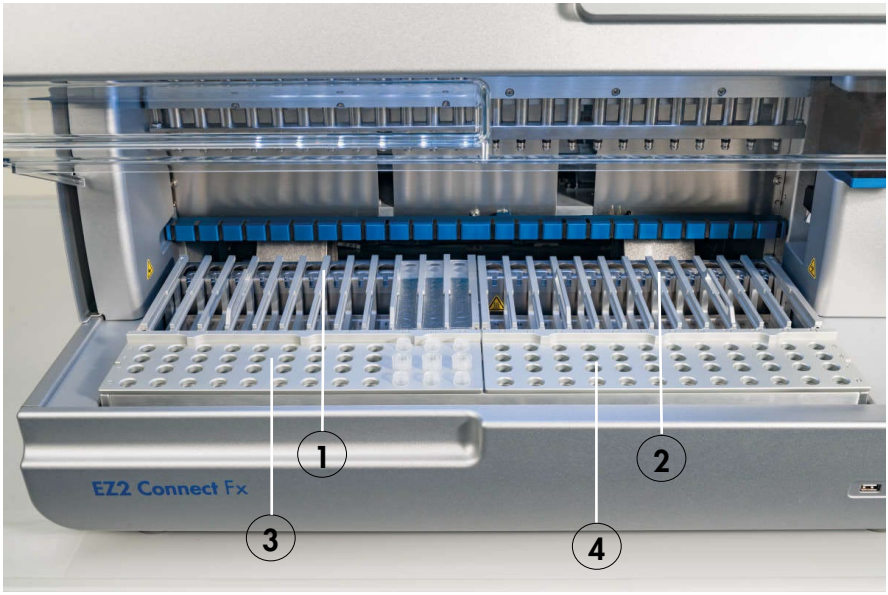


Figure 5. EZ2 Connect Worktable.

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*.

Protocol: EZ2 RNA FFPE Kit

Important notes before starting

- If any run has been performed before starting the EZ2 RNA FFPE protocol, run the "Cooling" protocol from the home screen first, in order to ensure that the heating block of the instrument is at room temperature to protect your RNA 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 four weeks or should be aliquoted and stored at –30 to –15°C for extended time periods while avoiding freeze–thaw cycles.
- Preheat a thermomixer at 56°C for use in step 2.
- Before loading reagent cartridges into the EZ2 Connect instrument, invert them 4 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. Place the FFPE sections in a 2 ml tube (supplied). Add 300 µl Paraffin Removal Solution, vortex vigorously for 10 s, and centrifuge briefly to bring the sample to the bottom of the tube.
2. Incubate for 3 min at 56°C.

   Optional: Vortex again after incubation, then briefly centrifuge the tube to remove drops from the inside of the lid and collect tissue at the bottom of the tube.
3. Turn on the EZ2 Connect instrument.
4. Tap "RNA" on the Applications panel, and then, select "RNA FFPE" and press **Next**.
5. Choose the "RNA FFPE" protocol and press **Next**.

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

6. Choose protocol and elution volume, and then press **Next**.

   Note: The choice of the "Standard" or "Fast" protocol version can influence the RNA quality. While "Standard" is recommended in most cases, the "Fast" protocol version may be beneficial for downstream applications relying more intact RNA fragments.

7. Select positions on the work deck according to the number of samples to be processed and press **Next**.
8. Enter sample IDs or press **Generate missing sample IDs**. Then press **Next**.
9. Open instrument hood. Load the EZ2 RNA FFPE reagent cartridges into respective positions of the EZ2 Connect Cartridge Rack as selected in step 7.
10. Load the EZ2 Connect Cartridge Rack into the EZ2 Connect instrument.
11. Remove the cap and place the 2 ml tube containing the sample from step 2 into position 11 of the reagent cartridge (positions are labelled by engravings). Press **Next**.
12. Remove caps of all tubes and prepare the EZ2 Connect Tip Rack (see Figure 4) as follows:
    - Position A: 1.5 ml tube with 10 µl DNase I stock solution + 16 µl DNase Booster
    - Position B: Tip holder with Filter Tip
    - Position C: Tip holder with Filter Tip
    - Position D: 1.5 ml tube
13. Place the EZ2 Connect Tip Rack into the EZ2 Connect instrument. Press **Next**.
14. Start the run according to the instructions on the instrument display.
15. The display will show "Protocol finished" when the run is completed. Select **Finish**.
16. Open the instrument hood. Remove the elution tube containing purified RNA from position D of the EZ2 Connect Tip Rack. Discard the used cartridge including the liquid waste.

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

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

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

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.
b) Magnetic particles not completely resuspended	Make sure to resuspend the magnetic particles thoroughly before loading the reagent cartridges into the holder.
General handling	
Error message in instrument display	Refer to the user manual supplied with your EZ2 Connect instrument.

Reference

1. Kunitz, M. (1950). Crystalline desoxyribonuclease; isolation and general properties; spectrophotometric method for the measurement of desoxyribonuclease activity. J. Gen. Physiol. **33**, 349–363.

Ordering Information

Product	Contents	Cat. no.
EZ2 RNA FFPE Kit (48)	For 48 preps: Paraffin Removal Solution, EZ2 RNA FFPE cartridge, Filter Tips and Holders, Tubes, RNase-free DNase, DNase Booster, and RNase-free water	959734
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		
RNase-Free DNase Set (50)	1500 Kunitz units RNase-free DNase I, RNase-free Buffer RDD, and RNase-free water for 50 RNA minipreps	79254
DNase Booster Buffer	Concentrated DNase treatment buffer for use with the QIAsymphony® RNA Kit (FFPE protocol), RNeasy FFPE Kit, or miRNeasy FFPE Kit	1064143
Filter Tips and Holders, EZ1® (50)	50 Disposable Filter-Tips, 50 Disposable Tip Holders; additional tips and holders for use with EZ1 Kits	994900

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	Changes
03/2022	Initial revision

Notes

Limited License Agreement for EZ2RNA FFPE and EZ2RNA FFPE UNG Kits

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