February 2022

EZ2® RNA/miRNA Tissue/Cells Handbook

For automated purification of total RNA including small RNAs using EZ2 Connect instruments

Sample to Insight

QIAGEN

Contents

Kit Contents

EZ2 RNA/miRNA Tissue/Cells Kit **Catalog no.** **No. of reactions**	**(48)** **959035** **48**
Buffer RLT†	45 ml
RNase-Free DNase I (lyophylized)	1500 units*
Proteinase K	1.4 ml
RNase-Free Water	2 x 1.9 ml
EZ2 RNA/miRNA Tissue/Cells Cartridges†	48
Disposable Tip Holders	50
Disposable Filter-Tips	50
Tubes 1.5 ml	50
Tubes 2 ml	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 6 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/miRNA Tissue/Cells 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, buffers and reagent cartridges are stable until the expiration date on the Q-Card and the kit label.

Intended Use

The EZ2 RNA/miRNA Tissue/Cells 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/miRNA Tissue/Cells 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/miRNA Tissue/Cells 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/miRNA Tissue/Cells Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

RNA analysis has become state of the art to analyze cell and tissue phenotypes. Interest in smaller RNA species, such as miRNA, has increased over the past years as researchers understand the regulatory role of small non-coding RNAs. The EZ2 RNA/miRNA Tissue/Cells Kit is designed for purification of total RNA — including miRNA and other small RNA — from cultured cells and various animal and human tissues.

The EZ2 RNA/miRNA Tissue/Cells Kit offers a phenol-free protocol to isolate high yields of total RNA including miRNA from up to 30 mg frozen and 15 mg stabilized tissue or up to 5 x 10^6 cells.

The EZ2 RNA/miRNA Tissue/Cells Kit provides a convenient, streamlined procedures for efficient, automated purification of RNA from tissue or cells including enzymatic removal of proteins and gDNA.

Principle and procedure

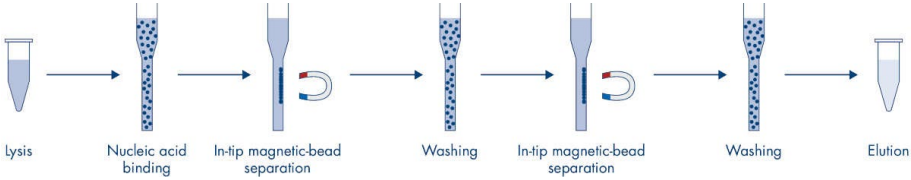
This protocol describes usage of the EZ2 RNA/miRNA Tissue/Cells Kit on the EZ2 Connect instruments. The EZ2 RNA/miRNA Tissue/Cells procedure starts with a manual lysis in buffer RLT, which protects RNA molecules. This is followed by a Proteinase K digestion under optimized conditions to ensure complete lysis of even difficult-to-lyse tissue and thereby release of RNA. All following steps are carried out on the EZ2 Connect instrument. After first binding to magnetic beads, RNA is treated with DNase to digest contaminating genomic DNA and is then rebound to magnetic particles. Contaminants that may interfere with following enzymatic reactions are removed in subsequent washing steps.

RNA is eluted in 50 or 100 µl 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

The EZ2 Connect instruments can perform all steps following lysis of the sample. This automation is based on magnetic particle technology and includes RNA binding, DNase digestion, washing, and elution. Up to 24 samples can be processed in a single run.

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 on the particles in the presence of a chaotropic salt. The particles are subsequently separated from the lysates using a magnet. An additional washing step removes any residual contaminants. Finally, RNA is efficiently eluted.



Real-time RT-PCR detection of miRNAs using the miRCURY® LNA® miRNA PCR system

In general, RT-PCR is recommended to accurately quantify yields of miRNA. The miRCURY LNA miRNA PCR system allows sensitive and specific quantification and profiling of miRNA expression using SYBR® Green-based or probe-based real-time PCR. Both the SYBR® Green detection-based miRCURY LNA miRNA PCR system and the probe-based miRCURY LNA miRNA Probe PCR System comprise all the required components to set up and conduct miRNA quantification and expression profiling, from conversion of RNA into cDNA to real-time PCR detection of miRNAs and straightforward data analysis. The systems both use the same miRCURY LNA RT Kit for generation of a universal first-strand cDNA synthesis – one cDNA reaction for all miRNAs. Each system then has their own dedicated master mix kit, the miRCURY LNA SYBR® Green PCR Kit and the miRCURY LNA miRNA Probe PCR Kit, as well as a broad variety of system-specific LNA-enhanced miRCURY LNA assay and panel products. The RNA Spike-In Kit enables quality control of the RNA isolation, cDNA synthesis, and PCR amplification steps of miRCURY LNA miRNA qPCR experiments.

For both systems, individual assays for mature miRNAs for a variety of different species can be ordered on GeneGlobe® (**www.qiagen.com/GeneGlobe**). Alternatively, for high-throughput and screening experiments, miRCURY LNA PCR Panels and miRCURY LNA Probe PCR Panels enable rapid profiling of the complete miRNome. Other Focus panels such as the miRCURY LNA miRNA Serum/Plasma Focus PCR Panel and the Serum/Plasma Focus Probe PCR Panel are available for the detection of mature miRNAs. Find out more about the miRCURY LNA miRNA PCR systems at **www.qiagen.com**.

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)
- Microcentrifuge with rotor for 2 ml tubes (up to 21,000 x *g*)
- Pipettors (2–1,000 µl)
- Microcentrifuge Tubes (e.g., Safe-Lock Tubes [Eppendorf, cat. no. 0030 120.086 or 0030 120.094])
- 14.3 M β-mercaptoethanol (β-ME, commercially available solutions are usually 14.3 M), alternatively dithiothreitol (DTT, 2 M stock solution)
- Sterile, RNase-free pipette tips
- Disposable gloves

  For animal tissues: RNAprotect® Tissue Reagent (see Ordering Information, page 34) or liquid nitrogen

- Equipment and tubes for disruption and homogenization (see page 14). Depending on the method chosen, one or more of the following are required:
  - TissueRuptor® II with TissueRuptor Disposable Probes (see Ordering Information, page 34)
  - TissueLyser II or TissueLyser LT (see Ordering Information, page 34)
  - Mortar and pestle
  - QIAshredder homogenizer (see Ordering Information, page 34)

Important Notes

Determining the correct amount of starting material — cells

The minimum amount of starting material is generally 100 cells, while the maximum amount depends on the RNA content of the cell type but should not exceed $5*10^6$ cells.

RNA content can vary greatly between cell types. The following examples illustrate how to determine the maximum amount of starting material:

- COS cells have high RNA content (approximately 35 µg RNA per 1 x 10^6 cells).
- HeLa cells have average RNA content (approximately 15 µg RNA per 1 x 10^6 cells).
- NIH/3T3 cells have low RNA content (approximately 10 µg RNA per 1 x 10^6 cells).

In general, we recommend starting with no more than 2–3 x 10^6 cells. Depending on RNA yield and purity, it may be possible to increase the cell number in subsequent preparations.

Counting cells is the most accurate way to quantify the amount of starting material. As a guide, the number of HeLa cells obtained in various culture vessels after confluent growth is given in Table 1.

Table 1. Growth area and number of HeLa cells in various culture vessels

Cell-culture vessel	Growth area (cm^2)*	Number of cells†
Multiwell-plates		
96-well	0.32–0.6	4–5 x 10^4
48-well	1	1 x 10^5
24-well	2	2.5 x 10^5
12-well	4	5 x 10^5
6-well	9.5	1 x 10^6
Dishes		
35 mm		1 x 10^6
60 mm		2.5 x 10^6
100 mm		7 x 10^6
145–150 mm		2 x 10^7

* Per well, if multiwell plates are used; varies slightly depending on the supplier.

† Cell numbers are given for HeLa cells (approximate length = 15 µm), assuming confluent growth. Cell numbers will vary for different kinds of animal cells, which vary in length from 10 to 30 µm.

Determining the correct amount of starting material — tissue

The maximum amount of tissue that can be processed depends on the RNA content of the tissue. In general, a maximum of 30 mg tissue can be processed with the EZ2 procedure. If you have no information about the nature of your starting material, we recommend starting with no more than 10 mg of tissue.

Weighing tissue is the most accurate way to quantify the amount of starting material. However, the following may be used as a guide. A 3 mm cube (volume, 27 mm^3) of most animal tissues weighs 25–35 mg.

Handling and storage of starting material

RNA is not protected after harvesting until the sample is treated with RNAprotect Cell Reagent (cultured cells only) or RNAprotect Tissue Reagent (animal tissues only), flash-frozen, or disrupted and homogenized in the presence of RNase-inhibiting or denaturing reagents.

Otherwise, unwanted changes in the gene expression profile will occur. It is therefore important that samples are immediately frozen in liquid nitrogen and stored at –90 to –65°C (animal tissues only), processed as soon as harvested, or immediately immersed in RNAprotect Cell Reagent or RNAprotect Tissue Reagent. Animal cells can be pelleted and then stored at –90 to –65°C until required for RNA purification.

An alternative to RNAprotect Tissue Reagent is Allprotect® Tissue Reagent, which provides immediate stabilization of DNA, RNA, and protein in tissue samples at room temperature.

The procedures for harvesting and RNA protection should be carried out as quickly as possible. Frozen samples should not be allowed to thaw during handling or weighing. After disruption and homogenization in Buffer RLT, samples can be stored at –90 to –65°C for months.

Disrupting and homogenizing starting material

Efficient disruption and homogenization of the starting material is a requirement for all total RNA purification procedures. Disruption and homogenization are 2 distinct steps:

- **Disruption**: Complete disruption of plasma membranes of cells and organelles is essential to release all the RNA contained in the sample. Different samples require different methods to achieve complete disruption. Incomplete disruption significantly reduces RNA yields.
- **Homogenization**: Homogenization is necessary to reduce the viscosity of the lysates produced by disruption. Homogenization shears high-molecular–weight genomic DNA and other high-molecular–weight cellular components to create a homogeneous lysate. In the EZ2 RNA/mRNA Tissue/Cells procedure, genomic DNA is removed by organic extraction, allowing to homogenize up to 3 x 10^6 cells by vortexing without additional homogenization. Incomplete homogenization results in inefficient binding of RNA to magnetic articles, significantly reducing RNA yields.

Some disruption methods simultaneously homogenize the sample, whereas others require an additional homogenization step. Table 2 gives an overview of different disruption and homogenization methods and is followed by a detailed description of each method. This information can be used as a guide to choose the appropriate methods for your starting material.

Table 2. Guide to methods of disruption and homogenization of samples

Sample	Disruption method	Homogenization method	Comments
Animal cells	Addition of lysis buffer	TissueRuptor II or QIAshredder homogenizer or syringe and needle or vortexing (≤3 x 10^6 cells)	If processing ≤3 x 10^6 cells, lysate can be homogenized by vortexing.
Animal tissues	TissueLyser II or TissueLyser LT	TissueLyser II or TissueLyser LT	The TissueLyser II and TissueLyser LT give results comparable to using a rotor–stator homogenizer
	TissueRuptor II	TissueRuptor II	Simultaneously disrupts and homogenizes
	Mortar and pestle	QIAshredder homogenizer or syringe and needle	The TissueRuptor II usually gives higher yields than mortar and pestle

Disruption and homogenization using the TissueRuptor II

The TissueRuptor II is a rotor–stator homogenizer that thoroughly disrupts and simultaneously homogenizes single animal tissue samples in the presence of lysis buffer in 15–90 seconds, depending on the toughness and size of the sample. The TissueRuptor II can also be used to homogenize cell lysates. The blade of the TissueRuptor II disposable probe rotates at a very high speed, causing the sample to be disrupted and homogenized by a combination of turbulence and mechanical shearing. For guidelines on disruption and homogenization of animal tissues using the TissueRuptor II, refer to the *TissueRuptor II Handbook*. For other rotor–stator homogenizers, please refer to suppliers' guidelines for further details.

Disruption and homogenization using the TissueLyser II or TissueLyser LT

In bead-milling, cells and tissues can be disrupted by rapid agitation in the presence of beads and lysis buffer. Disruption and simultaneous homogenization occur by the shearing and crushing action of the beads as they collide with the cells. Disruption efficiency is influenced by the following:

- Size and composition of beads
- Ratio of buffer to beads
- Amount of starting material
- Speed and configuration of the TissueLyser II or TissueLyser LT
- Disintegration time

Stainless-steel beads with a 3–7 mm diameter are optimal for use with animal tissues. All other disruption parameters must be determined empirically for each application. For guidelines on disruption and homogenization of tissues using the TissueLyser II and stainless-steel beads, refer to Appendix A (page 28). For other bead mills, please refer to the suppliers' guidelines for further details.

Disruption using a mortar and pestle

For disruption using a mortar and pestle, freeze the animal tissue immediately in liquid nitrogen and grind to a fine powder under liquid nitrogen. Transfer the suspension (tissue powder and liquid nitrogen) into a liquid-nitrogen–cooled, appropriately sized tube and allow the liquid nitrogen to evaporate without allowing the sample to thaw. Add lysis buffer and continue as quickly as possible with the homogenization according to one of the 2 methods below.

Note: Grinding the sample using a mortar and pestle will disrupt the sample but will not homogenize it. Homogenization must be performed afterwards.

Homogenization using QIAshredder homogenizers

Using QIAshredder homogenizers is a fast and efficient way to homogenize cell and tissue lysates without cross-contamination of samples. Up to 700 µl of lysate is loaded onto a QIAshredder spin column placed in a 2 ml collection tube and spun for 2 minutes at maximum speed in a microcentrifuge. The lysate is homogenized as it passes through the spin column.

Homogenization using a syringe and needle

Cell and tissue lysates can be homogenized using a syringe and needle. Lysate is passed through a 20-gauge (0.9 mm) needle attached to a sterile plastic syringe at least 5–10 times or until a homogeneous lysate is achieved. Increasing the volume of lysis buffer may be required to facilitate handling and minimize loss.

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 as it is 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 main features of EZ2 Connect instruments include the following:

- 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 1). 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 1. 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 holds 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 2. 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 3).

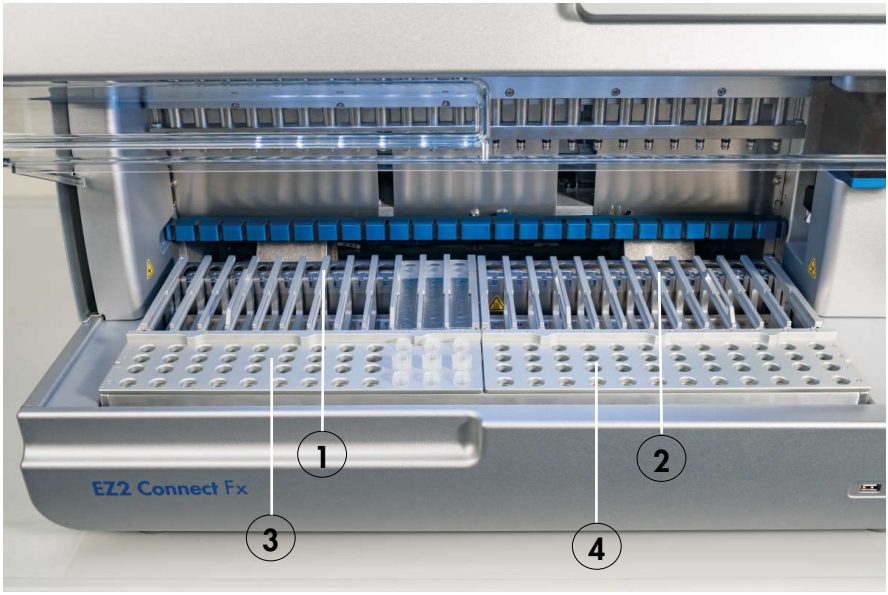


Figure 3. EZ2 Connect Worktable.

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/miRNA Tissue/Cells Kit

Important notes before starting

- 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
- The EZ2 RNA/miRNA Tissue/Cells Kit (cat. no. 959035) 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.
- If purifying RNA from cell lines rich in RNases or from tissue, we recommend adding either β-mercaptoethanol (β-ME) or 2 M dithiothreitol (DTT) to Buffer RLT before use (10 µl β-ME or 20 µl DTT per 1 ml Buffer RLT). Buffer RLT containing DTT or β-ME can be stored at room temperature for up to 1 month.
- Before adding DNase into the reagent cartridges and loading them into the EZ2 Connect instrument, invert the cartridges 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.
- In the following procedure, text marked with ● denotes RNA purification from cells, and text marked with ▲ denotes RNA purification from tissue samples.

RNA purification from cells

● Cells

1. Harvest cells as a cell pellet or, for cells grown in a monolayer, aspirate the cell-culture medium from the cell-culture vessel (up to 10 cm diameter).
2. Add 300 µl Buffer RLT to either the pellet or the cell-culture vessel, and homogenize (Table 1).

▲ Tissue samples

1. Add 300 µl Buffer RLT to tissue sample, and then disrupt and homogenize (Table 1).

2. Add 75 µl RNase-free Water and 25 µl Proteinase K, and mix and incubate for 10 min at room temperature. In the meantime, prepare the worktable as described below.

   Note: See Table 1 for the amount of starting material, and disruption and homogenization methods. Using more than the maximum recommended amount may result in reduced RNA yields and purity.

Procedure

1. Turn on the EZ2 Connect instrument.
2. **Important**: Ensure the heating block of the EZ2 Connect instrument is at room temperature.
3. Tap "RNA" on the Applications panel and then select the "RNA/miRNA from Tissue/Cells" and press **Next**.
4. Choose the "RNA/miRNA" protocol and press **Next**.
5. Choose elution volume and press **Next**.
6. Select positions on the work deck according to the number of samples to be processed and press **Next**.
7. Enter sample IDs or press **Generate missing sample IDs**. Then press **Next**.
8. Add 20 µl DNase to well 5 of the RNA/miRNA Tissue/Cells reagent cartridges and load it into the EZ2 Connect Cartridge Rack (Figure 2).
9. Open instrument hood. Load the EZ2 RNA/miRNA 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 caps of all tubes and prepare the EZ2 Connect Tip Rack as follows:

- Position A: 2 ml tube containing 400 µl lysate from step 4.
- Position C: Tip holder with Filter Tip
- Position D: 1.5 ml tube

12. Place the EZ2 Connect Tip Rack into the EZ2 Connect instrument. Press **Next**.
13. Start the run according to the instructions on the instrument display.
14. The display will show “Protocol finished” when the run is completed. Select **Finish**.
15. Open the instrument hood. Remove the elution tube containing purified nucleic acid 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.
16. 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
General handling	
a) 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.
c) Error message in instrument display	Refer to the user manual supplied with your EZ2 Connect instrument.
d) clogging of tips	Too much starting material, some tissues contain high amounts of gDNA (e.g., spleen or lung), which might lead to clogging the tips. Please reduce the input amount to reduce clogging issues.
RNA degraded	
a) Inappropriate handling of starting material	Ensure that tissue samples are properly stabilized and stored in RNAprotect Tissue Reagent. For frozen cell pellets or frozen tissue samples, ensure that they were flash-frozen immediately in liquid nitrogen and properly stored at "–90°C to –65°C. Perform the RNeasy procedure quickly, especially the first few steps. See Appendix A (page 28) and "Handling and storage of starting material" (page 13).
b) RNase contamination	Although all buffers in the EZ2 RNA/miRNA Tissue/Cells Kit have been tested and are guaranteed RNase-free, RNases can be introduced during use. Be certain not to introduce any RNases during the procedure or later handling. See Appendix A (page 28) for general remarks on handling RNA.
DNA contamination in downstream experiments	
a) Cell number too high	For some cell types, the efficiency of DNA removal may be reduced when processing very high cell numbers (containing more than 20 µg genomic DNA). If the eluted RNA contains substantial DNA contamination, try processing smaller cell numbers or perform DNase digestion of the eluted RNA followed by RNA cleanup.

	Comments and suggestions
b) Tissue has high DNA content	For certain tissues with extremely high DNA content (e.g., thymus), DNA may not be completely removed. Try using smaller samples (containing less than 20 µg genomic DNA) or perform DNase I digestion of the eluted RNA followed by RNA cleanup.
Low or no recovery of RNA	
a) Too much starting material	In subsequent preparations, reduce the amounts of starting material. It is essential to use the correct amount of starting material (see page 12).
b) Inefficient disruption and/or homogenization	See "Disrupting and homogenizing starting material" (page 14) for a detailed description of homogenization methods.

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.

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), use general laboratory reagents. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA,* followed by RNase-free water (see "Solutions", page 29), or rinse with chloroform* if the plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with detergent (e.g., 0.5% SDS),* rinse with RNase-free water, then rinse with ethanol (if the tanks are ethanol resistant) and allow to dry.

* 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.

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.* 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. 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 diethyl pyrocarbonate (DEPC)*, 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 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: RNeasy buffers are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

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

Storage of RNA

Purified RNA may be stored at –70°C to –15°C in RNase-free water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

The concentration of RNA should 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 be difficult to determine amounts photometrically. Small amounts of RNA can be quantified using quantitative RT-PCR or fluorometric quantification.

Spectrophotometric quantification of RNA

Using the QIAxpert UV/VIS Spectrophotometer for microvolume analysis

To determine the concentration of your RNA sample purified with RNeasy QIAGEN kit, use the corresponding RNeasy App on the QIAxpert®. For more information, see the QIAxpert product page (**www.qiagen.com/qiaxpert-system**).

Using a standard spectrophotometer

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 → 4 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 31), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity. 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 29). 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 assessment of RNA purity will be performed routinely, when using the QIAxpert with the corresponding RNeasy app. See the QIAxpert user manual for more information (**www.qiagen.com/qiaxpert-system/user manual**)

* 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 standard photometric measurements, 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, such as protein, that absorb in the UV spectrum. 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 when using pure water. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination.* 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 30).

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. RNeasy kits will, however, remove the vast majority of cellular DNA. gDNA Eliminator Solution helps to further reduce genomic DNA contamination; however, trace amounts of genomic DNA 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

* 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.

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

DNA cannot be avoided, we recommend using the QuantiTect Reverse Transcription Kit for reverse transcription. The kit integrates fast cDNA synthesis with rapid removal of genomic DNA contamination (see Ordering Information, page 34).

Integrity of RNA

The integrity and size distribution of total RNA purified with RNeasy Plus Universal Kits can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining* or by using the QIAxcel® system or Agilent® 2100 Bioanalyzer. Ribosomal RNAs should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S rRNA should be approximately 2:1. If the ribosomal bands or peaks of a specific sample are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the sample suffered major degradation either before or during RNA purification. As a useful measure of RNA integrity, the QIAxcel Advanced system and the Agilent 2100 Bioanalyzer provide an RNA integrity score (RIS) and an RNA integrity number (RIN), respectively. Ideally, the value should be close to 10, but in many cases (particularly with tissue samples), RNA quality is greatly influenced by how well the original sample was preserved.

* 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.

Ordering Information

Product	Contents	Cat. no.
EZ2 RNA/miRNA Tissue/Cells Kit (48)	For 48 preps: EZ2 RNA/miRNA Tissue/Cells cartridge, Filter Tips and Holders, Tubes, RNase-free DNase, Buffer RLT, Proteinase K	959035
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		
RNAprotect Tissue Reagent (50 ml)	For stabilization of RNA in 25 x 200 mg tissue samples: 50 ml RNAprotect Tissue Reagent	76104
RNAprotect Tissue Reagent (250 ml)	For stabilization of RNA in 125 x 200 mg tissue samples: 250 ml RNAprotect Tissue Reagent	76106
RNAprotect Cell Reagent (250 ml)	250 ml RNAprotect Cell Reagent	76526
Filter Tips and Holders, EZ1 (50)	50 Disposable Filter-Tips, 50 Disposable Tip Holders; additional tips and holders for use with EZ1, EZ1&2 and EZ2 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
02/2022	Initial revision

Notes

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