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RNeasy[®] FFPE Kit Instructions for Use

For purification of total RNA or total RNA, including miRNA, from formalin-fixed, paraffin-embedded tissue sections

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Kit Contents

RNeasy FFPE Kit	(50)
Catalog no.	73504
Number of preps	50
RNeasy MinElute Spin Columns (pink) (each in a 2 mL Collection Tube)	50
Collection Tubes (1.5 mL)	50
Collection Tubes (2 mL)	50
Buffer RBC*	45 mL
Buffer PKD	15 mL
Proteinase K	1.25 mL
RNase-Free DNase I (lyophilized)	1500 units
RNase-Free Water (for use with RNase-Free DNase I)	1.5 mL
DNase Booster Buffer	2 mL
Buffer RPE† (concentrate)	11 mL
RNase-Free Water	10 mL
Quick-Start Protocol	1

* Contains a guanidine salt. Not compatible with disinfectants containing bleach. See "Safety Information" on page 6.

† Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle, and described on page "Preparing Buffer RPE" on page 15, to obtain a working solution.

Intended Use

The RNeasy® FFPE Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention or treatment of a disease.

QIAcube® Connect is designed to perform fully automated purification of nucleic acids and proteins in molecular biology applications. The system is intended for use by professional users trained in molecular biological techniques and the operation of QIAcube Connect.

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.

Shipping and Storage

RNase-Free DNase I and RNeasy MinElute spin columns should be immediately stored at 2–8°C upon arrival. The buffers can be stored at room temperature (15–25°C). Under these conditions, the kit components can be kept for up to at least 9 months without any reduction in performance, if not otherwise stated on the label.

Proteinase K is supplied in a specially formulated storage buffer and is stable for at up to least 1 year after delivery when stored at room temperature. If longer storage is required or if ambient temperatures often exceed 25°C, we recommend storage at 2–8°C.

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.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of RNeasy FFPE Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

The RNeasy FFPE Kit is specially designed for purification of total RNA from formalin-fixed, paraffin-embedded (FFPE) tissue sections. By isolating RNA molecules longer than 70 nucleotides, the kit provides recovery of usable RNA fragments for applications, such as RT-PCR. However, if mainly fragments larger 200 nucleotides are required please follow the DV200 optimized protocol in Appendix B. Micro RNA (< 70 nt) can be recovered as well if binding conditions are adapted as described in the respective mi RNA protocol.

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 RNeasy FFPE Kit is optimized to reverse formaldehyde modification as much as possible without further RNA degradation, nucleic acids purified from FFPE samples should not be used in downstream applications that require full-length RNA. Some applications 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.

Staining of FFPE sections may also impair RNA quality and performance in downstream applications. This is especially true for many immunohistochemical staining protocols. However, cresyl violet staining has been shown to have minimal impact on performance in downstream real-time RT-PCR analysis and is recommended if staining of sections prior to RNA purification is necessary.

Principle and procedure

The RNeasy FFPE procedure uses well-established RNeasy technology for RNA purification. Specially optimized lysis conditions allow total RNA to be effectively purified from FFPE tissue sections. The DNase digestion step efficiently removes DNA contamination, including highly fragmented molecules.

Firstly, all paraffin is removed from freshly cut FFPE tissue sections by treating with Deparaffinization Solution or using an alternative deparaffinization method. Next, samples are incubated in an optimized lysis buffer, which contains Proteinase K, to release RNA from the sections. A short incubation at a higher temperature partially reverses formalin crosslinking of the released nucleic acids, improving RNA yield and quality, as well as RNA performance in downstream enzymatic assays. This is followed by DNase treatment that is optimized to eliminate all genomic DNA, including very small DNA fragments that are often present in FFPE samples after prolonged formalin fixation and/or long storage times. Next, the lysate is mixed with Buffer RBC. Ethanol is added to provide appropriate binding conditions for RNA, and the sample is then applied to an RNeasy MinElute spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. RNA is then eluted in a minimum of 14 µL of RNase-free water.

Solutions for FFPE research

QIAGEN's dedicated products for FFPE samples enable easy deparaffinization and efficient recovery of DNA, RNA, miRNA, and protein (see "Ordering Information" on page 56). Automation of FFPE sample prep can be performed using QIAcube Connect (see hereafter). Solutions for reliable downstream analysis include dedicated chemistry for PCR amplification of small fragments.

QIAGEN's comprehensive FFPE portfolio provides:

- Maximum data output with minimum sample consumption
- Technologies that reverse crosslinks for higher yields
- DNA, RNA, and protein purification without further compromising analyte integrity
- Optimized chemistries for analysis of lower quality FFPE analytes
- FFPE research data you can trust

Automated purification of RNA on the QIAcube instruments

Purification of RNA can be fully automated on the QIAcube Connect or the classic QIAcube. The innovative QIAcube instruments use advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using the QIAcube instruments follows the same steps as the manual procedure (i.e., lyse, bind, wash, and elute), enabling you to continue using the RNeasy FFPE Kit for purification of high-quality RNA.

The QIAcube instruments are preinstalled with protocols for purification of plasmid DNA, genomic DNA, RNA, viral nucleic acids and proteins, plus DNA and RNA cleanup. The range of protocols available is continually expanding, and additional QIAGEN protocols can be downloaded free of charge at www.qiagen.com/qiacubeprotocols.



Figure 1. QIAcube Connect

RNeasy DSP FFPE Procedure

FFPE tissue sections

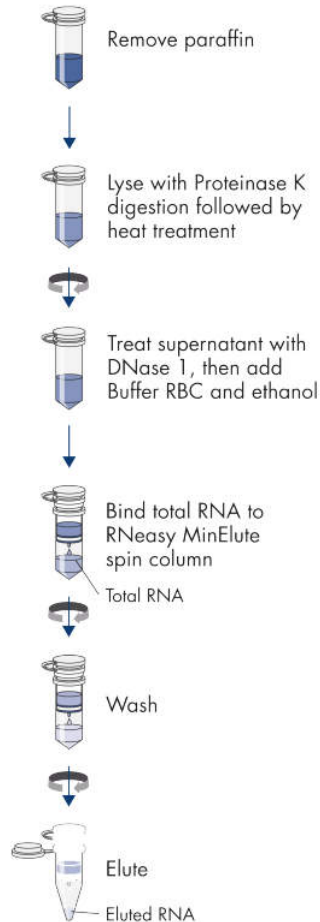


Figure 2. RNeasy FFPE procedure

Equipments 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, 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.

- Sterile, RNase-free pipet tips
- 1.5 mL or 2 mL centrifuge tubes
- Microcentrifuge (with rotor for 2 mL tubes)
- Vortexer
- 100% ethanol*
- For deparaffinization of FFPE tissue sections: Deparaffinization Solution (cat. no. 19093) or alternative reagent (e.g., heptane, xylene, limonene, CitriSolv). For details, see "Appendix A: Deparaffinization Methods" on page 39.
- Disposable gloves
- Heating block or water bath capable of incubation at 80°C

*Do not use denatured alcohol, which contains other substances, such as methanol or methylethylketone.

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:

- Use tissue samples less than 5 mm thick to allow complete penetration by formalin
- Fixate tissue samples in 4–10% neutral-buffered formalin as quickly as possible after surgical removal
- Use a maximum fixation time of 24 hours (longer fixation times lead to over-fixation and more severe nucleic acid fragmentation, resulting in poor performance in downstream assays)
- Thoroughly dehydrate samples prior to embedding
- Use low-melting paraffin for embedding

The starting material for RNA purification should be freshly cut sections of FFPE tissue, each with a thickness of up to 20 μm . Thicker sections may result in lower nucleic acid yields, even after prolonged incubation with Proteinase K. Up to 4 sections, each with a thickness of up to 10 μm and a surface area of up to 250 mm^2 , can be combined in one preparation. More than 4 sections can be combined if the total sum of the thickness of the sections is 40 μm or less (e.g., eight 5 μm thick sections), or if less than 30% of the surface area consists of tissue and the excess paraffin is removed using a scalpel prior to starting the protocol.

For tissues with particularly high DNA content, such as thymus, we recommend using fewer sections per preparation to avoid DNA contamination of the purified RNA.

If there is no information about the nature of your starting material, we recommend starting with no more than 2 sections per preparation. Depending on RNA yield and purity, it may be possible to use up to 4 sections in subsequent preparations.

For recommendations on using FFPE material other than microtome sections (e.g., core punches), please refer to www.qiagen.com/RNeasyFFPE.

Do not overload the RNeasy MinElute spin column, as this will significantly reduce RNA yield and quality.

Preparation of buffers

Preparing DNase I stock solution

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, do not open the vial. Instead, dissolve DNase using a needle and syringe as described below.

Prepare DNase I stock solution by dissolving the lyophilized DNase I (1500 Kunitz units) in 550 μ L RNase-free water. 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.

Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the vial.

Insoluble material may remain after dissolving DNase. Due to the production process, insoluble material may be present in the lyophilized DNase. This does not affect DNase performance.

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 $2-8^{\circ}\text{C}$ for up to 6 weeks. Do not refreeze the aliquots after thawing.

Preparing Buffer RPE

Add 4 volumes (44 mL) ethanol (96–100%) to the bottle containing 11 mL Buffer RPE concentrate. Tick the check box on the bottle label to indicate that ethanol has been added.

Note: Before starting the procedure, mix reconstituted Buffer RPE by shaking.

Protocol: Purification of Total RNA from FFPE Tissue Sections

Important points before starting

- If using the RNeasyFFPE Kit for the first time, read "Important Notes" on page 13.
- If working with RNA for the first time, read "Appendix C: General Remarks on Handling RNA" on page 49.
- Buffer RBC contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach. See "Safety Information" on page 6.
- Unless otherwise indicated, perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.
- Perform all centrifugation steps using a microcentrifuge placed at 15–25°C. If using a refrigerated microcentrifuge, set the temperature to 20–25°C; otherwise, significant cooling below 15°C may occur.
- In the procedure below, ▲ indicates the volumes to use if processing 1–2 sections per sample, while ■ indicates the volumes to use if processing >2 sections per sample.

Things to do before starting

1. If using Buffer RPE and RNase-Free DNase I for the first time, reconstitute them as described in "Preparation of buffers" on page 14.
2. Equilibrate all buffers to room temperature (15–25°C). Mix reconstituted Buffer RPE by shaking.
3. Set a thermal mixer, heat block, or water bath to 56°C for use in step 5 and step 9. To

reduce waiting time, set a second thermal mixer, heat block, or water bath to 80°C for use in step 9.

Procedure

1. Using a scalpel, trim excess paraffin off the sample block.
2. Cut sections 5–20 µm thick.

If the sample surface has been exposed to air, discard the first 2–3 sections.

3. Immediately place the sections in a ▲ 1.5 or 2 mL or ■ 2 mL microcentrifuge tube (not supplied), and close the lid.
4. Add ▲ 160 µL or ■ 320 µL Deparaffinization Solution, vortex vigorously for 10 s, and centrifuge briefly to bring the sample to the bottom of the tube.

Deparaffinization Solution is not supplied with the RNeasy FFPE Kit and should be ordered separately (cat. no. 19093). If using an alternative deparaffinization method, see "Appendix A: Deparaffinization Methods" on page 39 for further details.

5. Incubate at 56°C for 3 min, then allow to cool at room temperature (15–25°C).

If too little Deparaffinization Solution is used or if too much paraffin is carried over with the sample, the Deparaffinization Solution may become waxy or solid after cooling. If this occurs, add additional Deparaffinization Solution and repeat the 56°C incubation.

6. Add ▲ 150 µL or ■ 240 µL Buffer PKD, and mix by vortexing.
7. Centrifuge for 1 min at 11,000 x g (10,000 rpm).
8. Add 10 µL Proteinase K to the lower, clear phase. Mix gently by pipetting up and down.
9. Incubate at 56°C for 15 min, then at 80°C for 15 min.

If a heating block without a shaking function is used, briefly mix by vortexing every 3–5 min. If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block has reached 80°C.

Note: Complete digestion of tissue by Proteinase K is not required for maximum RNA yield; however, the 80°C incubation step is crucial.

Important: Ensure that the heating block has reached 80°C before starting the 15 min incubation. The 15 min incubation at 80°C is critical for reversal of crosslinks and optimal RNA performance in downstream applications, such as real-time RT-PCR.

The incubation at 80°C in Buffer PKD partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented RNA but may also result in slightly lower C_T values in downstream applications, such as real-time RT-PCR.

10. Transfer the lower, uncolored phase into a new 2 mL microcentrifuge tube.
11. Incubate on ice for 3 min. Then, centrifuge for 15 min at 20,000 x g (13,500 rpm).
12. Transfer the supernatant to a new microcentrifuge tube (not supplied), taking care not to disturb the pellet.

The pellet contains insoluble tissue debris, including crosslinked DNA.

If processing more than 2 sections, a larger tube may be necessary.

13. Add DNase Booster Buffer equivalent to a tenth of the total sample volume (approx. ▲ 16 µL or ■ 25 µL) and 10 µL DNase I stock solution. Mix by inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.

Note: DNase I is supplied lyophilized and should be reconstituted as described in "Preparing DNase I stock solution" on page 14.

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

14. Incubate at room temperature for 15 min.
15. Add ▲ 320 µL or ■ 500 µL Buffer RBC to adjust binding conditions, and mix the lysate thoroughly.
16. Add ▲ 720 µL or ■ 1200 µL ethanol (100%) to the sample, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 17.

Precipitates may be visible after addition of ethanol. This does not affect the procedure.

17. Transfer 700 µL of the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2 mL collection tube (supplied). Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.*

Reuse the collection tube in step 18.

18. Repeat step 17 until the entire sample has passed through the RNeasy MinElute spin column.

Reuse the collection tube in step 19.

19. Add 500 µL Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.

Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added before use as described in "Preparing Buffer RPE" on page 15.

Reuse the collection tube in step 20.

20. Add 500 µL Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the collection tube with the flow-through.

*Flow-through contains Buffer RBC and is therefore not compatible with bleach. See "Safety Information" on page 6.

Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

21. Place the RNeasy MinElute spin column in a new 2 mL collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the collection tube with the flow-through.

To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

Note: It is important to dry the spin column membrane because residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

22. Place the RNeasy MinElute spin column in a new 1.5 mL collection tube (supplied). Add 14–30 μ L RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

Elution with smaller volumes of RNase-free water leads to higher total RNA concentrations, but lower RNA yields.

The dead volume of the RNeasy MinElute spin column is 2 μ L: elution with 14 μ L RNase-free water results in a 12 μ L eluate.

Protocol: Purification of Total RNA, Including miRNA, from FFPE Tissue Sections

Important points before starting

- If using the RNeasyFFPE Kit for the first time, read "Important Notes" on page 13.
- If working with RNA for the first time, read "Appendix C: General Remarks on Handling RNA" on page 49.
- Buffer RBC contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach. See "Safety Information" on page 6.
- Unless otherwise indicated, perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.
- Perform all centrifugation steps using a microcentrifuge placed at 15–25°C. If using a refrigerated microcentrifuge, set the temperature to 20–25°C; otherwise, significant cooling below 15°C may occur.
- In the procedure below, ▲ indicates the volumes to use if processing 1–2 sections per sample, while ■ indicates the volumes to use if processing >2 sections per sample.

Things to do before starting

1. If using Buffer RPE and RNase-Free DNase I for the first time, reconstitute them as described in "Preparation of buffers" on page 14.
2. Equilibrate all buffers to room temperature (15–25°C). Mix reconstituted Buffer RPE by shaking.
3. Set a thermal mixer, heat block, or water bath to 56°C for use in step 5 and step 9. To

reduce waiting time, set a second thermal mixer, heat block, or water bath to 80°C for use in step 9.

Procedure

1. Using a scalpel, trim excess paraffin off the sample block.
2. Cut sections 5–20 µm thick. If the sample surface has been exposed to air, discard the first 2–3 sections.
3. Immediately place the sections in a ▲ 1.5 mL or 2 mL or ■ 2 mL microcentrifuge tube (not supplied), and close the lid.
4. Add ▲ 160 µL or ■ 320 µL Deparaffinization Solution, vortex vigorously for 10 s, and centrifuge briefly to bring the sample to the bottom of the tube.

Deparaffinization Solution is not supplied with the miRNeasy FFPE Kit and should be ordered separately (cat. no. 19093). If using an alternative deparaffinization method, see "Appendix A: Deparaffinization Methods" on page 39 for further details.

5. Incubate at 56°C for 3 min, then allow to cool at room temperature.
6. Add ▲ 150 µL or ■ 240 µL Buffer PKD, and mix by vortexing.
7. Centrifuge for 1 min at 11,000 x g (10,000 rpm).
8. Add 10 µL Proteinase K to the lower, clear phase. Mix gently by pipetting up and down.
9. Incubate at 56°C for 15 min, then at 80°C for 15 min. If a heating block without a shaking function is used, briefly mix by vortexing every 3–5 min. If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block has reached 80°C.

Important: Ensure that the heating block has reached 80°C before starting the 15 min incubation. The 15 min incubation at 80°C is critical for optimal RNA performance in downstream applications, such as real-time RT-PCR. The incubation at 80°C in Buffer PKD

partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented RNA, but may also result in slightly lower C_T values in downstream applications, such as real-time RT-PCR.

10. Transfer the lower, clear phase into a new 2 mL microcentrifuge tube. If processing more than 2 sections, a larger tube may be necessary.
11. Incubate on ice for 3 min. Then centrifuge for 15 min at 20,000 x g (13,500 rpm).
12. Transfer the supernatant to a new microcentrifuge tube (not supplied) taking care not to disturb the pellet. The pellet contains insoluble tissue debris, including crosslinked DNA.
13. Add DNase Booster Buffer equivalent to a tenth of the total sample volume (approximately ▲ 16 μ L or ■ 25 μ L) and 10 μ L DNase I stock solution. Mix by inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.

Note: DNase I is supplied lyophilized and should be reconstituted as described in "Preparing DNase I stock solution" on page 14.

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

14. Incubate at room temperature for 15 min.
15. Add ▲ 320 μ L or ■ 500 μ L Buffer RBC to adjust binding conditions, and mix the lysate thoroughly.
16. Add ▲ 1120 μ L or ■ 1750 μ L ethanol (100%) to the sample, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 17.

Precipitates may be visible after addition of ethanol. This does not affect the procedure.

17. Transfer 700 μ L of the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2 mL collection tube (supplied). Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.

Reuse the collection tube in step 18.

18. Repeat step 17 until the entire sample has passed through the RNeasy MinElute spin column.

Reuse the collection tube in step 19.

19. Add 500 μ L Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.

Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added before use as described in "Preparing Buffer RPE" on page 15.

Reuse the collection tube in step 20.

20. Add 500 μ L Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the collection tube with the flow-through.

Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

21. Place the RNeasy MinElute spin column in a new 2 mL collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the collection tube with the flow-through.

To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

Note: It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

22. Place the RNeasy MinElute spin column in a new 1.5 mL collection tube (supplied). Add 14–30 μ L RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

Note: Elution with smaller volumes of RNase-free water leads to higher total RNA concentrations, but lower RNA yields. The dead volume of the RNeasy MinElute spin column is 2 μ L: elution with 14 μ L RNase-free water results in a 12 μ L eluate.

Protocol: Purification of Total RNA from Microdissected FFPE Tissue Sections

This protocol is for the purification of total RNA from microdissected, formalin-fixed samples. Laser-microdissected tissue specimens present a particular challenge for molecular analysis, as nucleic acids must be purified from very small amounts of starting material. In addition, fixation and staining steps may compromise the integrity of RNA. For staining: cresyl violet has been shown to have a minimal impact on RNA quality.

This protocol is not suitable for RNA purification from microdissected cryosections. For these applications, we recommend the RNeasy Plus Micro Kit (cat. no. 74034).

A wide range of equipment and consumables for sectioning, staining, and microdissection of specimens is available from Leica® (www.leica-microsystems.com) and P.A.L.M. Microlaser Technologies (www.palm-mikrolaser.com).

Important points before starting

- If using the RNeasyFFPE Kit for the first time, read "Important Notes" on page 13.
- If working with RNA for the first time, read "Appendix C: General Remarks on Handling RNA" on page 49.
- Buffer RBC contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach. See "Safety Information" on page 6.
- Unless otherwise indicated, perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.
- Perform all centrifugation steps using a microcentrifuge placed at 15–25°C. If using a

refrigerated microcentrifuge, set the temperature to 20–25°C; otherwise, significant cooling below 15°C may occur.

Things to do before starting

1. If using Buffer RPE and the RNase-Free DNase I for the first time, reconstitute them as described in "Preparation of buffers" on page 14.
2. Equilibrate all buffers to room temperature (15–25°C). Mix reconstituted Buffer RPE by shaking.
3. Set a thermal mixer or heat block or water bath to 56°C for use in step 4. Preferably set another thermal mixer or heat block or water bath to 80°C to avoid waiting times in step 4.
4. In case the sample material has not been deparaffinized prior to dissection, collect the dissected material into Deparaffinization Solution, transfer to a 1.5 mL microcentrifuge tube (not supplied), and adjust the volume of Deparaffinization Solution to 160 µL.
5. Proceed with the "Protocol: Purification of Total RNA from FFPE Tissue Sections" on page 16, starting with step 5 on page 16 and follow the instructions for using lower volumes (indicated by ▲). For alternative deparaffinization methods, see "Appendix A: Deparaffinization Methods" on page 39.

Procedure

1. After microdissection, place the sample into a collection vessel containing an appropriate volume of Buffer PKD. The volume depends on the collection vessel used for microdissection but should not be greater than 65 µL (Leica instruments) or 150 µL (other instruments).
2. If necessary, transfer the sample and buffer to a larger vessel (e.g., 1.5 or 2 mL tube). Adjust the volume to 150 µL if necessary by adding buffer PKD.

3. Add 10 μL Proteinase K. Mix gently by pipetting up and down.
4. Incubate at 56°C for 15 min, then at 80°C for 15 min.

If a heating block without a shaking function is used, briefly mix by vortexing every 3–5 min. If using only one heating block, leave the sample at room temperature (15–25°C) after the 56°C incubation until the heating block has reached 80°C.

Important: Ensure that the heating block has reached 80°C before starting the 15 min incubation. The 15 min incubation at 80°C is critical for reversal of crosslinks and optimal RNA performance in downstream applications, such as real-time RT-PCR.

Note: The incubation at 80°C in Buffer PKD partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented RNA but may also result in slightly lower CT values in downstream applications, such as real-time RT-PCR.

5. Incubate on ice for 3 min. Then, centrifuge for 15 min at 20,000 \times g (13,500 rpm).
6. Transfer the supernatant to a new microcentrifuge tube (not supplied) taking care not to disturb the pellet.

The pellet contains insoluble tissue debris, including crosslinked DNA.

7. Add DNase Booster Buffer equivalent to a tenth of the total sample volume (approx. 16 μL) and 10 μL DNase I stock solution. Mix by inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.

Note: DNase I is supplied lyophilized and should be reconstituted as described in "Preparing DNase I stock solution" on page 14.

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

8. Incubate at room temperature for 15 min.
9. Add 320 μL Buffer RBC to adjust binding conditions, and mix the lysate thoroughly.

10. Add 720 μL ethanol (100%) to the sample, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 11.

Note: Precipitates may be visible after addition of ethanol. This does not affect the procedure.

11. Transfer 700 μL of the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2 mL collection tube (supplied). Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.*

Reuse the collection tube in step 12.

12. Repeat step 11 until the entire sample has passed through the RNeasy MinElute spin column.

Reuse the collection tube in step 13.

13. Add 500 μL Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the flow-through.

Reuse the collection tube in step 14.

Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added before use as described in "Preparing Buffer RPE" on page 15.

14. Add 500 μL Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the collection tube with the flow-through.

Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

*Flow-through contains Buffer RBC and is therefore not compatible with bleach. See "Safety Information" on page 6.

15. Place the RNeasy MinElute spin column in a new 2 mL collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the collection tube with the flow-through.

Note: To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

Note: It is important to dry the spin column membrane because residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

16. Place the RNeasy MinElute spin column in a new 1.5 mL collection tube (supplied). Add 14–30 μ L RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

Elution with smaller volumes of RNase-free water leads to higher total RNA concentrations, but lower RNA yields.

The dead volume of the RNeasy MinElute spin column is 2 μ L; elution with 14 μ L RNase-free water results in a 12 μ L eluate.

Protocol: Purification of Total RNA, Including miRNA, from Microdissected FFPE Tissue Sections

This protocol is for the purification of total RNA, including miRNA, from microdissected, formalin-fixed samples. Laser-microdissected tissue specimens present a particular challenge for molecular analysis, as nucleic acids must be purified from very small amounts of starting material. In addition, fixation and staining steps may compromise the integrity of RNA.

This protocol is not suitable for RNA purification from microdissected cryosections. For these applications, we recommend the RNeasy Plus Micro Kit (cat. no. 74034).

A wide range of equipment and consumables for sectioning, staining and microdissection of specimens is available from Leica® (www.leica-microsystems.com) and P.A.L.M. Microlaser Technologies (www.palm-mikrolaser.com).

Important points before starting

- If using the RNeasyFFPE Kit for the first time, read "Important Notes" on page 13.
- If working with RNA for the first time, read "Appendix C: General Remarks on Handling RNA" on page 49.
- Buffer RBC contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach. See "Safety Information" on page 6.
- Unless otherwise indicated, perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.
- Perform all centrifugation steps using a microcentrifuge placed at 15–25°C. If using a

refrigerated microcentrifuge, set the temperature to 20–25°C; otherwise, significant cooling below 15°C may occur.

Things to do before starting

- If using Buffer RPE and the RNase-Free DNase I for the first time, reconstitute them as described in "Preparation of buffers" on page 14.
- Equilibrate all buffers to room temperature (15–25°C). Mix reconstituted Buffer RPE by shaking.
- Set a thermal mixer, heating block or water bath to 56°C for use in step 4 and step 8. If possible, set a second thermal mixer, heating block or water bath to 80°C for use in step 8.

Procedure

1. After microdissection, place the sample into a collection vessel containing an appropriate volume of Deparaffinization Solution. The volume depends on the collection vessel used for microdissection, but should not be greater than 65 µL (Leica instruments) or 300 µL (other instruments).

Note: Deparaffinization Solution is not supplied with the miRNeasy FFPE Kit and should be ordered separately (cat. no. 19093). If using an alternative deparaffinization method, see "Appendix A: Deparaffinization Methods" on page 39 for further details.

2. If necessary, transfer the sample and buffer to a larger vessel (e.g., 1.5 or 2 mL tube). The total volume should be at least 160 µL. Adjust the volume to 160 µL if necessary by adding Deparaffinization Solution.
3. Vortex vigorously for 10 s, and centrifuge briefly to bring the sample to the bottom of the tube.
4. Incubate at 56°C for 3 min, then allow to cool at room temperature.
5. Add 150 µL Buffer PKD, and mix by vortexing.

6. Centrifuge for 1 min at 11,000 x *g* (10,000 rpm).
7. Add 10 µL Proteinase K to the lower, clear phase. Mix gently by pipetting up and down.
8. Incubate at 56°C for 15 min, then at 80°C for 15 min. If a heating block without a shaking function is used, briefly mix by vortexing every 3–5 min. If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block has reached 80°C.

Important: Ensure that the heating block has reached 80°C before starting the 15 min incubation. The 15 min incubation at 80°C is critical for optimal RNA performance in downstream applications, such as real-time RT-PCR. The incubation at 80°C in Buffer PKD partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented RNA, but may also result in slightly lower C_T values in downstream applications, such as real-time RT-PCR.

9. Transfer the lower, clear phase into a new 2 mL microcentrifuge tube.
10. Incubate on ice for 3 min. Then centrifuge for 20 min at 20,000 x *g* (13,500 rpm).
11. Transfer the supernatant to a new microcentrifuge tube (not supplied) taking care not to disturb the pellet. The pellet contains insoluble tissue debris, including crosslinked DNA.
12. Add DNase Booster Buffer equivalent to a tenth of the total sample volume (approximately 16 µL) and 10 µL DNase I stock solution. Mix by inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.

Note: DNase I is supplied lyophilized and should be reconstituted as described in "Preparing DNase I stock solution" on page 14.

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

13. Incubate at room temperature for 15 min.
14. Add 320 µL Buffer RBC to adjust binding conditions, and mix the lysate thoroughly.

15. Add 1120 μL ethanol (100%) to the sample, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 16. Precipitates may be visible after addition of ethanol. This does not affect the procedure.

16. Transfer 700 μL of the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2 mL collection tube (supplied). Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.*

Reuse the collection tube in step 17.

17. Repeat step 16 until the entire sample has passed through the RNeasy MinElute spin column.

Reuse the collection tube in step 18.

18. Add 500 μL Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the flow-through.

Reuse the collection tube in step 19.

Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added before use as described in "Preparing Buffer RPE" on page 15.

19. Add 500 μL Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the collection tube with the flow-through.

Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

*Flow-through contains Buffer RBC and is therefore not compatible with bleach. See "Safety Information" on page 6.

20. Place the RNeasy MinElute spin column in a new 2 mL collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the collection tube with the flow-through.

Note: To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

Note: It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

21. Place the RNeasy MinElute spin column in a new 1.5 mL collection tube (supplied). Add 14–30 μL RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

Elution with smaller volumes of RNase-free water leads to higher total RNA concentrations, but lower RNA yields.

The dead volume of the RNeasy MinElute spin column is 2 μL ; elution with 14 μL RNase-free water results in a 12 μL eluate.

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 at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx (for contact information, visit www.qiagen.com).

Comments and suggestions	
Clogged RNeasy MinElute spin column	
a. Too much starting material	Reduce the amount of starting material. It is essential to use the correct amount of starting material (see "Important Notes" on page 13).
b. Centrifugation temperature too low	The centrifugation temperature should be 15–25°C. Some centrifuges may cool to below 15°C even when set at 20°C. This can cause formation of precipitates that can clog the RNeasy MinElute spin column. If this happens, set the centrifugation temperature to 25°C. Warm the ethanol-containing sample to 37°C before transferring it to the RNeasy MinElute spin column.
Low RNA yield	
a. Poor quality of starting material	Samples that were fixed for over 20 h or stored for very long periods may contain very little usable RNA. Sections that were mounted on microscope slides may yield very little usable RNA due to prolonged exposure to air.
b. Too much starting material	Overloading the RNeasy MinElute spin column significantly reduces nucleic acid yields. Reduce the amount of starting material (see "Important Notes" on page 13).
c. Insufficient deparaffinization or sample contains too much paraffin	If using another method, consider using Deparaffinization Solution (cat. no. 19093).
d. RNA still bound to RNeasy MinElute spin column membrane	Repeat RNA elution, but incubate the RNeasy MinElute spin column on the benchtop for 10 min with RNase-free water before centrifuging.

Low A_{260}/A_{280} value

Water used to dilute nucleic acid for A_{260}/A_{280} measurement

Use 10 mM Tris-Cl, pH 7.5, not water, to dilute the sample before measuring purity (see "Appendix D: Storage, Quantification and Determination of Quality of RNA" on page 52).

DNA contamination in downstream experiments

- a. Too much starting material

For some tissue types, the efficiency of DNA removal may be reduced when processing very high amounts. If the eluted RNA contains substantial DNA contamination, try processing fewer tissue sections per preparation.

- b. Tissue has high DNA content

When processing very large amounts of tissues rich in DNA (e.g., thymus), the DNA may not be completely digested. Repeat the purification procedure using fewer tissue sections.

Alternatively, if using the RNA in real-time two-step RT-PCR, synthesize the cDNA using the QuantiTect® Reverse Transcription Kit (cat. no. 205311), which integrates reverse transcription with elimination of genomic DNA contamination.

RNA does not perform well in downstream assays

- a. RNA fragmented or blocked due to formaldehyde modification

The 80°C incubation in the RNeasy FFPE procedure is crucial for optimal RNA performance in reverse transcription and other enzymatic downstream applications. Ensure that the incubation temperature is maintained at 80°C throughout the entire 15 min incubation time.

Although the 80°C incubation removes some of the formaldehyde modifications, RNA purified from FFPE sections is not an optimal template for enzymatic reactions. We recommend using only random primers or gene-specific primers for cDNA synthesis. We also recommend keeping amplicons as short as possible for PCR (<500 nucleotides).

Comments and suggestions

- | | |
|--|--|
| b. Ethanol carryover | During the second wash with Buffer RPE, be sure to centrifuge at $\geq 8000 \times g$ ($\geq 10,000$ rpm) for 2 min at 15–25°C to dry the RNeasy MinElute spin column membrane. After centrifugation, carefully remove the column from the collection tube so that the column does not contact the flow-through. Then, be sure to place the column in a new collection tube and centrifuge at full speed for 5 min. |
| c. Salt carryover during RNA elution | Ensure that Buffer RPE is at room temperature (15–25°C). |
| d. Reverse transcription with insufficient amount of RNA | <p>Most reverse transcriptases are intended for use with approx. 1 µg RNA. If performing reverse transcription with very small amounts of RNA, we recommend using the Sensiscript® RT Kit (cat. no. 205211), which is specially designed for highly sensitive reverse transcription using <50 ng RNA.</p> <p>If performing one-step RT-PCR or quantitative, real-time RT-PCR, we recommend using the QuantiFast® Probe RT-PCR Plus Kit (cat. no. 204482), which integrates reverse transcription with elimination of genomic DNA contamination.</p> |

Appendix A: Deparaffinization Methods

Prior to nucleic acid purification from FFPE samples, paraffin must be removed to enable exposure of the sample to Proteinase K. We recommend using Deparaffinization Solution (cat. no. 19093) for this purpose. Alternatively, heptane, xylene, limonene, CitriSolv, or melting can be used, as described below.

Deparaffinization using heptane and methanol

Deparaffinization with heptane and methanol is done without any additional washing steps. This procedure usually provides good results in RNA applications.

In the procedure below, ▲ indicates the volumes to use if processing 1–2 sections per sample, while ■ indicates the volumes to use if processing >2 sections per sample.

1. Add 500 μL heptane, vortex vigorously for 10 s, and incubate for 10 min at room temperature (15–25°C).
2. Add 25 μL methanol, vortex vigorously for 10 s, and centrifuge for 2 min at 9000 $\times g$ (11,000 rpm).
3. Carefully remove the supernatant by pipetting without disturbing the pellet. Carefully remove any residual heptane/methanol using a fine pipet tip.
4. Keep the lid open, and air dry the pellet for 5 min at room temperature.
5. Add ▲ 150 μL or ■ 240 μL Buffer PKD, and mix by vortexing.
6. Add 10 μL Proteinase K. Mix gently by pipetting up and down.
7. Incubate at 56°C for 15 min, then at 80°C for 15 min.

If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block has reached 80°C.

Note: Complete digestion of tissue by Proteinase K is not required for maximum RNA yield; however, the 80°C incubation step is crucial.

Important: Ensure that the heating block has reached 80°C before starting the 15 min incubation. The 15 min incubation at 80°C is critical for reversal of crosslinks and optimal RNA performance in downstream applications, such as real-time RT-PCR.

The incubation at 80°C in Buffer PKD partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented RNA but may also result in slightly lower CT values in downstream applications, such as real-time RT-PCR.

8. Proceed to step 11 of the protocol for FFPE tissue sections (page "Protocol: Purification of Total RNA from FFPE Tissue Sections" on page 16) or step 5 of the protocol for microdissected FFPE tissue sections (page "Protocol: Purification of Total RNA from Microdissected FFPE Tissue Sections" on page 26).

Deparaffinization using xylene, limonene, or CitriSolv

Paraffin is first dissolved in xylene, limonene, or CitriSolv. After precipitation of the sample and removal of the supernatant, residual xylene is removed by washing with ethanol. This procedure is commonly used when purifying RNA from FFPE samples for use in different applications.

In the procedure below, ▲ indicates the volumes to use if processing 1–2 sections per sample, while ■ indicates the volumes to use if processing >2 sections per sample.

1. Add 1 mL xylene, limonene, or CitriSolv. Vortex vigorously for 10 s, and centrifuge at full speed for 2 min.

2. Carefully remove the supernatant by pipetting without disturbing the pellet.
3. Add 1 mL ethanol (96–100%) to the pellet, mix by vortexing, and centrifuge at full speed for 2 min.

The ethanol extracts residual xylene from the sample.

4. Carefully remove the supernatant by pipetting without disturbing the pellet. Carefully remove any residual ethanol using a fine pipet tip.
5. Keep the lid open, and incubate at room temperature (15–25°C) or at up to 37°C. Incubate for 10 min or until all residual ethanol has evaporated.
6. Add ▲ 150 µL or ■ 240 µL Buffer PKD, and mix by vortexing.
7. Add 10 µL Proteinase K. Mix gently by pipetting up and down.
8. Incubate at 56°C for 15 min, then at 80°C for 15 min.

If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block has reached 80°C.

Note: Complete digestion of tissue by Proteinase K is not required for maximum RNA yield; however, the 80°C incubation step is crucial.

Important: Ensure that the heating block has reached 80°C before starting the 15 min incubation. The 15 min incubation at 80°C is critical for reversal of crosslinks and optimal RNA performance in downstream applications, such as real-time RT-PCR.

The incubation at 80°C in Buffer PKD partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented RNA but may also result in slightly lower CT values in downstream applications, such as real-time RT-PCR.

9. Proceed to step 11 of the protocol for FFPE tissue sections (page "Protocol: Purification of Total RNA from FFPE Tissue Sections" on page 16) or step 5 of the protocol for microdissected FFPE tissue sections (page "Protocol: Purification of Total RNA from

Microdissected FFPE Tissue Sections" on page 26).

Deparaffinization using melting

When paraffin is melted and cooled, it forms a solid layer on an aqueous phase. This layer can then be pierced with a pipet tip to continue the purification protocol. It is necessary to avoid clogging of the pipet tips with paraffin.

In the procedure below, ▲ indicates the volumes to use if processing 1–2 sections per sample, while ■ indicates the volumes to use if processing >2 sections per sample.

1. Add ▲ 150 μ L or ■ 240 μ L Buffer PKD, and mix by vortexing.
2. Heat for 3 min at 55°C, vortex vigorously for 10 s, then centrifuge at full speed for 2 min.
3. Add 10 μ L Proteinase K. Mix gently by pipetting up and down. Pierce the solid paraffin layer with a pipet tip to add the Proteinase K.
4. Incubate at 56°C for 15 min, then at 80°C for 15 min.

If using only one heating block, leave the sample at room temperature (15–25°C) after the 56°C incubation until the heating block has reached 80°C.

Note: Complete digestion of tissue by Proteinase K is not required for maximum RNA yield; however, the 80°C incubation step is crucial.

Important: Ensure that the heating block has reached 80°C before starting the 15 min incubation. The 15 min incubation at 80°C is critical for reversal of crosslinks and optimal RNA performance in downstream applications, such as real-time RT-PCR.

The incubation at 80°C in Buffer PKD partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented RNA, but may also result in slightly lower CT values in downstream applications, such as real-time RT-PCR.

5. Proceed to step 11 of the protocol for FFPE tissue sections (page "Protocol: Purification of Total RNA from FFPE Tissue Sections" on page 16) or step 5 of the protocol for microdissected FFPE tissue sections (page "Protocol: Purification of Total RNA from Microdissected FFPE Tissue Sections" on page 26).

Appendix B: DV200 Optimized Purification of Total RNA from FFPE Tissue Sections

The DV200 index is a metric commonly used to assess RNA integrity in formalin-fixed, paraffin-embedded (FFPE) samples. It represents the percentage of RNA fragments longer than 200 nucleotides in a sample.

While the DV200 index can provide some insights into RNA quality, it is important to understand its limitations. The ability of an isolation kit to bind and purify smaller RNA fragments can impact the DV200 index. If the isolation kit effectively captures and retains smaller fragments during the purification process, it can potentially decrease the DV200 index. However, using isolation kits that selectively only capture longer RNA fragments while washing away shorter fragments can result in the loss of a significant portion of the sample. This loss of material can be problematic, especially when working with limited or precious samples as smaller fragments can still contain valuable information and contribute to meaningful results. For improvement of the DV200 compared to the standard protocol, please follow the below protocol.

Important points before starting

- If using the RNeasyFFPE Kit for the first time, read "Important Notes" on page 13.
- If working with RNA for the first time, read "Appendix C: General Remarks on Handling RNA" on page 49.
- Buffer RBC contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach. See "Safety Information" on page 6.
- Unless otherwise indicated, perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.

- Perform all centrifugation steps using a microcentrifuge placed at 15–25°C. If using a refrigerated microcentrifuge, set the temperature to 20–25°C; otherwise, significant cooling below 15°C may occur.
- In the procedure below, ▲ indicates the volumes to use if processing 1–2 sections per sample, while ■ indicates the volumes to use if processing >2 sections per sample.

Things to do before starting

1. If using Buffer RPE and RNase-Free DNase I for the first time, reconstitute them as described in "Preparation of buffers" on page 14.
2. Equilibrate all buffers to room temperature (15–25°C). Mix reconstituted Buffer RPE by shaking.
3. Set a thermal mixer, heat block, or water bath to 56°C for use in step 5 and step 9. To reduce waiting time, set a second thermal mixer, heat block, or water bath to 80°C for use in step 9.

Procedure

1. Using a scalpel, trim excess paraffin off the sample block.
2. Cut sections 5–20 µm thick. If the sample surface has been exposed to air, discard the first 2–3 sections.
3. Immediately place the sections in a ▲ 1.5 or 2 mL or ■ 2 mL microcentrifuge tube (not supplied), and close the lid.
4. Add ▲ 160 µl or ■ 320 µl Deparaffinization Solution, vortex vigorously for 10 s, and centrifuge briefly to bring the sample to the bottom of the tube.

Note: Deparaffinization Solution is not supplied with the RNeasy FFPE Kit and should be ordered separately (cat. no. 19093). If using an alternative deparaffinization method, see "Appendix A: Deparaffinization Methods" on page 39 for further details.

5. Incubate at 56°C for 3 min, then allow to cool at room temperature (15–25°C). If too little Deparaffinization Solution is used or if too much paraffin is carried over with the sample, the Deparaffinization Solution may become waxy or solid after cooling. If this occurs, add additional Deparaffinization Solution and repeat the 56°C incubation.
6. Add ▲ 150 µl or ■ 240 µl Buffer PKD, and mix by vortexing.
7. Centrifuge for 1 min at 11,000 x g (10,000 rpm).
8. Add 10 µl Proteinase K to the lower, clear phase. Mix gently by pipetting up and down.
9. Incubate at 56°C for 15 min, then at 80°C for 15 min. If a heating block without a shaking function is used, briefly mix by vortexing every 3–5 min. If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block has reached 80°C.

Note: Complete digestion of tissue by Proteinase K is not required for maximum RNA yield; however, the 80°C incubation step is crucial.

Important: Ensure that the heating block has reached 80°C before starting the 15 min incubation. The 15 min incubation at 80°C is critical for reversal of crosslinks and optimal RNA performance in downstream applications, such as real-time RT-PCR. The incubation at 80°C in Buffer PKD partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented RNA but may also result in slightly lower C_T values in downstream applications, such as real-time RT-PCR.

10. Transfer the lower, uncolored phase into a new 2 mL microcentrifuge tube.
11. Incubate on ice for 3 min. Then, centrifuge for 15 min at 20,000 x g (13,500 rpm).
12. Transfer the supernatant to a new microcentrifuge tube (not supplied), taking care not to disturb the pellet. The pellet contains insoluble tissue debris, including crosslinked DNA. If processing more than 2 sections, a larger tube may be necessary.

13. Add DNase Booster Buffer equivalent to a tenth of the total sample volume (approx. ▲ 16 µl or ■ 25 µl) and 10 µl DNase I stock solution. Mix by inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.

Note: DNase I is supplied lyophilized and should be reconstituted as described in "Preparing DNase I stock solution" on page 14.

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

14. Incubate at room temperature for 15 min.
15. Add ▲ 320 µl or ■ 500 µl Buffer RBC to adjust binding conditions, and mix the lysate thoroughly.
16. Add ▲ 300 µl or ■ 500 µl ethanol (100%) to the sample, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 17. Precipitates may be visible after addition of ethanol. This does not affect the procedure.
17. Transfer 700 µl of the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2 mL collection tube (supplied). Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.*

Reuse the collection tube in step 18.

18. Repeat step 17 until the entire sample has passed through the RNeasy MinElute spin column.

Reuse the collection tube in step 19.

19. Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.

*Flow-through contains Buffer RBC and is therefore not compatible with bleach. See "Safety Information" on page 6.

Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added before use as described in "Preparing Buffer RPE" on page 15.

Reuse the collection tube in step 20.

20. Add 500 μ L Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the collection tube with the flow-through.

Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

21. Place the RNeasy MinElute spin column in a new 2 mL collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the collection tube with the flow-through.

Note: To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

Note: It is important to dry the spin column membrane because residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

22. Place the RNeasy MinElute spin column in a new 1.5 mL collection tube (supplied). Add 14–30 μ L RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

Elution with smaller volumes of RNase-free water leads to higher total RNA concentrations, but lower RNA yields.

The dead volume of the RNeasy MinElute spin column is 2 μ L; elution with 14 μ L RNase-free water results in a 12 μ L eluate.

Appendix C: 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., pipets and electrophoresis tanks), use of commercially available RNase removal reagents is recommended. RNase contamination can alternatively be removed using general laboratory reagents. To decontaminate plasticware, rinse with 0.1 M NaOH, 1

mM EDTA* followed by RNase-free water (see "Solutions" below), 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, 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 presence of Tris buffers and decomposes rapidly into ethanol and CO₂. When preparing Tris

*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.

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.

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

Storage of RNA

Purified RNA may be stored at -30°C to -15°C or -70°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 (www.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 ($A_{260}=1 \rightarrow 44 \mu\text{g/mL}$). 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 hereafter (see "Purity of RNA" on the facing page), 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" on page 50). 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 μ L

Dilution = 10 μ L of RNA sample + 490 μ L of 10 mM Tris-Cl, * pH 7.0 (1/50 dilution)

Measure absorbance of diluted sample in a 1 mL cuvette (RNase-free)

$$A_{260} = 0.2$$

$$\begin{aligned}\text{Concentration of RNA sample} &= 44 \text{ } \mu\text{g/mL} \times A_{260} \times \text{dilution factor} \\ &= 44 \text{ } \mu\text{g/mL} \times 0.2 \times 50 \\ &= 440 \text{ } \mu\text{g/mL}\end{aligned}$$

$$\begin{aligned}\text{Total amount} &= \text{concentration} \times \text{volume in milliliters} \\ &= 440 \text{ } \mu\text{g/mL} \times 0.1 \text{ mL} \\ &= 44 \text{ } \mu\text{g of RNA}\end{aligned}$$

Purity of RNA

The ratio of the readings at 260 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

^{*}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.

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.* 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 $\mu\text{g/mL}$ RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Spectrophotometric quantification of RNA" on page 52).

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 RNeasy 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

*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.

www.qiagen.com/GeneGlobe). For two-step, real-time RT-PCR assays where amplification of genomic DNA cannot be avoided, the QuantiTect Reverse Transcription Kit (cat. 205311) provides fast cDNA synthesis with removal of genomic DNA contamination. For one-step, real-time RT-PCR the QuantiFast Probe RT-PCR Plus Kit (cat. no. 204482) integrates reverse transcription and removal of genomic DNA contamination.

Integrity of RNA

The integrity and size distribution of total RNA purified with the RNeasy 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.

Ordering Information

Product	Contents	Cat. no.
RNeasy FFPE Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	73504
Deparaffinization Solution	2 x 8 mL Deparaffinization Solution	19093
Accessories		
Collection Tubes (2 mL)	1000 Collection Tubes (2 mL)	19201
QIAGEN Proteinase K (2 mL)	2 mL (>600 mAU/mL, solution)	19131
QIAGEN Proteinase K (10 mL)	10 mL (>600 mAU/mL, solution)	19133
Buffer RPE (concentrate, 55 mL)	55 mL concentrated wash buffer for use with RNeasy, miRNeasy and AllPrep® Kits; requires addition of ethanol	1018013
QIAcube Connect — for fully automated nucleic acid extraction with QIAGEN spin-column kits		
QIAcube Connect*	Instrument, connectivity package, 1-year warranty on parts and labor	Inquire
Starter Pack, QIAcube	Filter-tips, 200 µL (1024), 1000 µL filter-tips (1024), 30 mL reagent bottles (12), rotor adapters (240), elution tubes (240), rotor adapter holder	990395
RELATED PRODUCTS		
AllPrep DNA/RNA FFPE Kit (50)	50 RNeasy MinElute Spin Columns, 50 QIAamp MinElute Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	80234
QIAamp DNA FFPE Advanced Kit (50)	For 50 preps: QIAamp UCP MinElute Columns, collection tubes, Deparaffinization Solution, Proteinase K, RNase A, RNase-free water and buffers	56604

Product	Contents	Cat. no.
QIAamp DNA FFPE Advanced UNG Kit (50)	For 50 preps: Uracil-N-glycosylase, QIAamp UCP MinElute columns, collection tubes, Deparaffinization Solution, Proteinase K, RNase A, RNase-free water and buffers	56704
QIAamp DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute Columns, Proteinase K, Buffers, Collection Tubes (2 mL)	56404
REPLI-g® FFPE Kit (25)	DNA Polymerase, Buffers and Reagents for 25 x 50 µL whole genome amplification reactions	150243
REPLI-g FFPE Kit (100)	DNA Polymerase, Buffers and Reagents for 100 x 50 µL whole genome amplification reactions	150245
EpiTect® Fast DNA Bisulfite Kit (50)	For 50 preps: Bisulfite Solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA, and Buffers	59824
Qproteome® FFPE Tissue Kit (20)	For 20 protein preparations from FFPE tissue samples: Extraction Buffer, Collection Tubes, Collection Tube Sealing Clips	37623
QuantiNova Probe RT-PCR Kit (500)	For 500 x 20 µL reactions: 3 x 1.7 mL QuantiNova Probe RT-PCR Master Mix, 100 µL QuantiNova Probe RT Mix, 100 µL Internal Control RNA, 500 µL Yellow Template Dilution Buffer, 1 mL ROX Reference Dye, 2 x 1.9 µL RNase-Free Water	208354
RT² PreAMP cDNA Synthesis Kit (12)	For twelve 20 µL reactions: 5x gDNA Elimination Buffer, 5x Reverse Transcription Buffer 3, cDNA Synthesis Enzyme Mix, RNase Inhibitor, Primer and External Control Mix, RNase-Free Water, RT² PreAMP PCR Master Mix, Side Reaction Reducer	330451

Product	Contents	Cat. no.
RT ² PreAMP Primer Mixes	Pathway-Focused Primer Mixes for all RT ² Profiler PCR Arrays	Varies

* All QIAcube Connect instruments are provided with a region-specific connectivity package, including tablet and equipment necessary to connect to the local network. Further, QIAGEN offers comprehensive instrument service products, including service agreements, installation, introductory training and preventive subscription. Contact your local sales representative to learn about your options.

† Visit www.qiagen.com/GeneGlobe to search for and order this product.

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Document Revision History

Revision	Description
01/2020	Updated text and ordering information for QIAcube Connect.
07/2021	Updated the URL referring to the RNeasy FFPE Kit product webpage. Corrected the page and step numbers referring to the protocol for FFPE tissue sections and the protocol for microdissected FFPE tissue sections. Updated the text regarding removal of RNase contamination from bench surfaces, nondisposable plasticware, and laboratory equipment in Appendix C. Updated the Ordering Information section.
02/2025	Clarification regarding storage period on Shipping and Storage section Updated Introduction section to add information regarding larger fragments Inclusion of new protocols: <ul style="list-style-type: none">• Purification of total RNA, including miRNA, from FFPE tissue sections• Purification of total RNA including miRNA, from microdissected FFPE tissue sections Added new Appendix regarding DV200 optimized purification of total RNA Updated Ordering Information section to for additional related products

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