May 2018

miRNeasy FFPE Handbook

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

Sample to Insight

QIAGEN

Contents

Handbook Revision History

Document	Changes	Date
HB-0374-002	Updated kit contents, removed reference to heated orbital shaker and emphasized importance of removing DEPC.	June 2013
HB-0374-003	Changes to comply with GHS regulation, throughout the document.	June 2015
HB-0374-004	Update into Sample-to-Insight branding and edit into revised style. Importance of vortexing when heating block without shaking function is used emphasized in protocols. Redundant step in Appendix A removed, ordering Information updated.	May 2018

Kit Contents

miRNeasy FFPE Kit	**(50)**
Catalog no.	**217504**
No. 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	2

* Contains a guanidine salt. Not compatible with disinfectants containing bleach. See page 6 for safety information.

† Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle, and described on page 14, to obtain a working solution.

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 at least 9 months without any reduction in performance.

Proteinase K is supplied in a specially formulated storage buffer and is stable for at least 1 year after delivery when stored at room temperature (15–25°C). If longer storage is required or if ambient temperatures often exceed 25°C, we recommend storage at 2–8°C.

Intended Use

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

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

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

Buffer RBC contains guanidine hydrochloride which can form highly reactive compounds when combined with bleach. If liquid containing this buffer is spilt, clean with 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.

Quality Control

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

Introduction

The miRNeasy FFPE Kit is specially designed for purification of total RNA, including miRNA, from formalin-fixed, paraffin-embedded (FFPE) tissue sections. By isolating RNA molecules longer than approximately 18 nucleotides, the kit provides recovery of usable RNA fragments, including miRNA and other small RNA, for applications such as RT-PCR.

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 miRNeasy FFPE Kit is optimized to reverse as much formaldehyde modification 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 miRNeasy 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 a 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, including miRNA, binds to the membrane and contaminants are efficiently washed away. Total RNA including miRNA is then eluted in a minimum of 14 µl of RNase-free water.

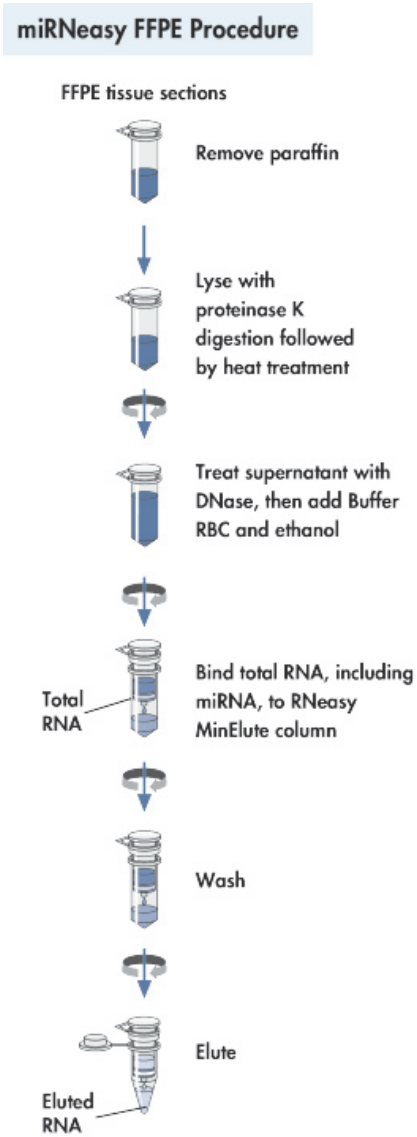

Figure 1. miRNeasy FFPE workflow.

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", page 40). Automation of FFPE sample prep can be performed using the QIAcube®. 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
- Prep 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

Purification of RNA from FFPE samples can be automated on the QIAcube. The innovative QIAcube uses 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 follows the same steps as the manual procedure (i.e., lyse, DNase treat, bind, wash and elute), enabling you to continue using the miRNeasy FFPE Kit for purification of high-quality RNA. For more information about the automated procedure, see the relevant protocol sheet available at **www.qiagen.com/MyQIAcube**.

The QIAcube is 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/MyQIAcube**.

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.

- 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 further details, see Appendix A, page 28.
- 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 in order 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.

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 µl RNase-free water (provided). 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 –15 to –30°C for up to 9 months. Thawed aliquots can be stored at 2–8°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, Including miRNA, from FFPE Tissue Sections

Important points before starting

- If using the miRNeasy FFPE Kit for the first time, read "Important Notes" (page 12).
- If working with RNA for the first time, read Appendix B (page 32).
- Buffer RBC contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach. See page 6 for safety information.
- 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

- If using Buffer RPE and RNase-Free DNase I for the first time, reconstitute them as described in "Preparation of buffers" (page 13).
- 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 5 and step 9. If possible, set a second thermal mixer, heating 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 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 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" (page 13).

    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 ▲ 1120 µl or ● 1750 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 ≥8000 x *g* (≥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 ≥8000 x *g* (≥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" (page 14).

    Reuse the collection tube in step 20.

* Flow-through contains Buffer RBC and is therefore not compatible with bleach. See page 6 for safety information.

20. Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥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).

    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 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.

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 miRNeasy FFPE Kit for the first time, read "Important Notes" (page 12).
- If working with RNA for the first time, read Appendix B (page 28).
- Buffer RBC contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach. See page 6 for safety information.
- 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" (page 13).
- 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).

   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 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" (page 13).

    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 ≥8000 x *g* (≥10,000 rpm). Discard the flowthrough.*

    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 ≥8000 x *g* (≥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" (page 14).

19. Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥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.

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.

    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).

* Flow-through contains Buffer RBC and is therefore not compatible with bleach. See page 6 for safety information.

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**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and/or protocols in this handbook or sample and assay technologies (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 page 12).
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 page 12).

		Comments and suggestions
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 C, page 35).
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.

	Comments and suggestions
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. If using the RNA for detection of miRNA or other noncoding RNA, we recommend the miScript PCR System. miScript Primer Assays will generally not detect genomic DNA, due to the design of the miScript PCR System. However, it is still important to avoid DNA contamination by not using too many sections per preparation. In contrast, miScript Precursor Assays are sensitive to genomic DNA contamination. When using miScript Precursor Assays for precursor miRNA detection, we recommend performing a "no RT" (no reverse transcription) control to determine whether DNA contamination is present in the reaction. If using the RNA for mRNA detection 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 miRNeasy 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 minute 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. For quantification of miRNA and other noncoding RNA by real-time RT-PCR, we recommend using the miScript PCR System. For real-time RT-PCR analysis of longer RNAs, we recommend using only random primers or gene-specific primers for cDNA synthesis. We also recommend keeping amplicons as short as possible for PCR (<150 nucleotides).

	Comments and suggestions
b) Ethanol carryover	During the second wash with Buffer RPE, be sure to centrifuge at ≥8000 x *g* (≥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	If using the RNA for detection of miRNA or other noncoding RNA with the miScript PCR System (see "Ordering Information", page 40), 10 pg–1 µg RNA is a sufficient starting amount. If necessary, use more tissue sections for RNA purification. If using >1 µg RNA, scale up the reaction linearly to the appropriate volume. If using the RNA for mRNA detection by real-time RT-PCR, it is usually necessary to use approximately 1 µg RNA with most reverse transcriptases. 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. 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.

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 volume to use if processing 1–2 sections per sample, while ● indicates the volume 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 x *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 (15–25°C).
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.

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 CT values in downstream applications such as real-time RT-PCR.

8. Proceed to step 11 of the protocol for FFPE tissue sections (page 16) or step 10 of the protocol for microdissected FFPE tissue sections (page 21).

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 volume to use if processing 1–2 sections per sample, while ● indicates the volume 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.

   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. Proceed to step 11 of the protocol for FFPE tissue sections (page 16) or step 10 of the protocol for microdissected FFPE tissue sections (page 21).

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 volume to use if processing 1–2 sections per sample, while ● indicates the volume to use if processing >2 sections per sample.

1. Add ▲ 150 µl or ● 240 µl 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 µl 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 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 CT values in downstream applications such as real-time RT-PCR.

5. Proceed to step 11 of the protocol for FFPE tissue sections (page 16) or step 10 of the protocol for microdissected FFPE tissue sections (page 21).

Appendix B: 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. In order 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 RNaseKiller (cat. no 2500080) from 5 PRIME (www.5prime.com) 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", page 33), or rinse with chloroform* if the plasticware is chloroform-resistant. To

* 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.

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 should be purchased RNase-free or treated with 0.1% DEPC. We recommend purchasing RNase-free water. This is because, if trace amounts of DEPC remain after autoclaving buffer, purine residues in RNA might be modified by carbethoxylation and performance of enzymatic reactions such as PCR may be negatively affected. Therefore, residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for at least 15 minutes.

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. 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 buffers, use RNase-free water to dissolve Tris to make the appropriate buffer.

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

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

Storage of RNA

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

Quantification of RNA

The concentration of RNA 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 the QIAxpert®, QIAxcel® Advanced System or Agilent® 2100 bioanalyzer, quantitative RT-PCR or fluorometric quantification.

Spectrophotometric quantification of RNA

To ensure significance, A_{260} readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml (A_{260} = 1 → 44 µg/ml). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH.* As discussed below (see "Purity of RNA", page 37), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

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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 33). 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 RNA sample + 490 µl 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

* 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.

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination.* 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 35).

To assess the purity of RNA (A_{260}/A_{280}) we recommend using the QIAxpert. The QIAxpert is an innovative µ-volume UV/Vis spectrophotometer that overcomes the limitations of classic spectrophotometry and purity assessment using absorbance ratios. Using reference spectra of known contaminants, a state-of-the-art software algorithm on the QIAxpert instrument performs a deconvolution of measured UV/Vis spectra according to the Beer Lambert law for mixtures, stating that the absorption spectrum of a mixture is a linear combination of the spectra of its individual constituents. This feature, known as Spectral Content Profiling (SCP), allows dye-free and easy differentiation between DNA, RNA and other UV/Vis absorbing contaminants in complex biological samples).

* 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 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 RNeasy Kits 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.

For quantification of miRNA by real-time RT-PCR, we recommend the miScript PCR System (see "Ordering Information", page 40). miScript Primer Assays will generally not detect genomic DNA, due to the design of the miScript PCR System. In contrast, miScript Precursor Assays are sensitive to genomic DNA contamination.

For quantification of mRNA by real-time RT-PCR, 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 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 miRNeasy FFPE Kit can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining* or by using the QIAxcel 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. Although electrophoretic analysis is not influenced significantly by formaldehyde modifications present on RNA isolated from FFPE samples, performance of RNA from FFPE samples in enzymatic assays will be significantly inferior compared to RNA of similar integrity from non-FFPE samples.

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Ordering Information

Product	Contents	Cat. no.
miRNeasy FFPE Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	217504
Deparaffinization Solution	2 x 8 ml Deparaffinization Solution	19093
Related products		
QIAamp® DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	56404
RNeasy FFPE Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	73504
GeneRead™ DNA FFPE Kit (50)	For 50 preps: QIAamp MinElute Columns, Collection Tubes, Deparaffinization Solution, Uracil-N-Glycosylase, RNase-Free Water, RNase A, and Buffers	180134
AllPrep® DNA/RNA FFPE Kit	50 RNeasy MinElute Spin Columns, 50 QIAamp MinElute Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	80234

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.

Notes

Notes

Limited License Agreement for miRNeasy FFPE Kit

Use of this product signifies the agreement of any purchaser or user of the product to the following terms:

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