Absolutely RNA FFPE Kit

INSTRUCTION MANUAL

Catalog #400809, #400811

Revision B

For In Vitro Use Only

400809-12

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Absolutely RNA FFPE Kit

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MATERIALS PROVIDED

Materials Provided[a]	Catalog #400809	Catalog #400811
Components Shipped at Room Temperature		
Deparaffinization Reagent (d-limonene) (**Shipped separately**)	150 ml	—
RNA Binding Buffer	35 ml	35 ml
β-Mercaptoethanol (β-ME) (14.2 M)	300 µl	300 µl
High Salt Wash Buffer (1.67×)	24 ml	24 ml
Low Salt Wash Buffer (5×)	17 ml	17 ml
Elution Buffer (10 mM Tris-HCl, pH 7.5)	3 ml	3 ml
RNase-free DNase I (lyophilized)	2600 U	2600 U
DNase Reconstitution Buffer	300 µl	300 µl
DNase Digestion Buffer	1.5 ml	1.5 ml
Prefilter Spin Cups (blue) and 2-ml receptacle tubes	50 each	50 each
RNA-Binding Spin Cups (white) and 2-ml receptacle tubes	50 each	50 each
1.5-ml collection tubes	50	50
Components Shipped on Dry Ice		
Proteinase K (20 mg/ml)	0.5 ml	0.5 ml
Proteinase K Digestion Buffer	5 ml	5 ml
Stratagene QPCR Human Reference Total RNA (1 µg/µl)	25 µg	25 µg

[a] Sufficient reagents are provided to isolate total RNA from 50 tissue samples.

Note *This kit is shipped in multiple parts—three parts for catalog # 400809 (two room temperature and one dry ice shipments) and two parts for catalog #400811 (one room temperature and one dry ice shipment). Ensure that all parts have been received before initiating experiments.*

STORAGE CONDITIONS

Deparaffinization Reagent: Store at room temperature.
Proteinase K and Proteinase K Digestion Buffer: Store at –20°C.
Stratagene QPCR Human Reference Total RNA: Store at –80°C.
Other Components: Upon, receipt, store the remaining components at room temperature. After first use, store the components under the following conditions:

β-Mercaptoethanol: Once opened, store at 4°C.
RNase-Free DNase I: Once reconstituted, store at –20°C.
All Other Components: Store at room temperature.

Caution *Guanidine thiocyanate in the RNA Binding Buffer and High-Salt Wash Buffer is an irritant. The Deparaffinization Reagent is combustible and should be stored away from heat. See the Material Data Safety Sheets, available at http://www.stratagene.com/MSDS for precautions.*

Revision B

ADDITIONAL MATERIALS REQUIRED

Sulfolane [Sigma (Catalog #T22209)]
Ethanol [100%, 90%, and 70% (v/v)], prepared using DNase-, RNase-free H_2O
Catalog #400811 only: A suitable substitute for the Deparaffinization Reagent (d-limonene) is Hemo-De, available from Scientific Safety Solvents (Keller, TX, catalog #HD150A)

INTRODUCTION

The Absolutely RNA FFPE Kit allows purification of QRT-PCR-ready total RNA from formalin-fixed, paraffin-embedded (FFPE) tissue samples.

Human surgical tissue samples are routinely fixed with formalin and then embedded in paraffin for long-term preservation. The extensive histopathology archive of formalin-fixed, paraffin-embedded (FFPE) tissue samples represents an extremely valuable resource for retrospective studies of gene expression patterns in diseased tissues. Limitations on using these samples for mRNA expression studies have been imposed by the significant modification and degradation of RNA during tissue fixation,[1, 2] resulting in poor yield of RNA obtained when using standard RNA isolation methods. Using methods that reliably deliver a sufficient yield of RNA, the recovered RNA is suitable for gene expression analysis using QRT-PCR assays.[3–6] The Absolutely RNA FFPE kit protocol has been specifically optimized to remove the modifications made to the RNA molecule during tissue fixation that would otherwise render the RNA useless in downstream QRT-PCR. As a result, using this purification method increases the yield of RNA from FFPE tissues that can serve as template for QRT-PCR.

Overview of the Method

The Absolutely RNA FFPE kit uses a simple, reliable, phenol-free protocol to isolate RNA from FFPE tissue sections. See Figure 1 for an overview of this procedure. First, tissue sections are deparaffinized with d-limonene and then washed in ethanol-based solutions to remove the Deparaffinization Reagent. Next, the sections are treated with Proteinase K to solubilize the fixed tissue and release the nucleic acids into solution. Finally, total RNA is isolated using the Absolutely RNA Kit. The RNA isolation protocol begins with suspending the sample in RNA Binding Buffer, containing a strong protein denaturant (the chaotropic salt guanidine thiocyanate), which prevents ribonuclease (RNase) degradation of the RNA. The sample is filtered using a micro-spin cup, and then loaded onto a second micro-spin cup containing a silica-based fiber matrix. The nucleic acids in the sample bind to the fiber matrix and then the DNA component is efficiently removed from the matrix-bound sample by on-column DNase I treatment. The immobilized RNA is washed to remove contaminants, and total RNA is recovered in a final volume of 30 µl. The isolated pure RNA is ready for gene expression analysis by QRT-PCR.

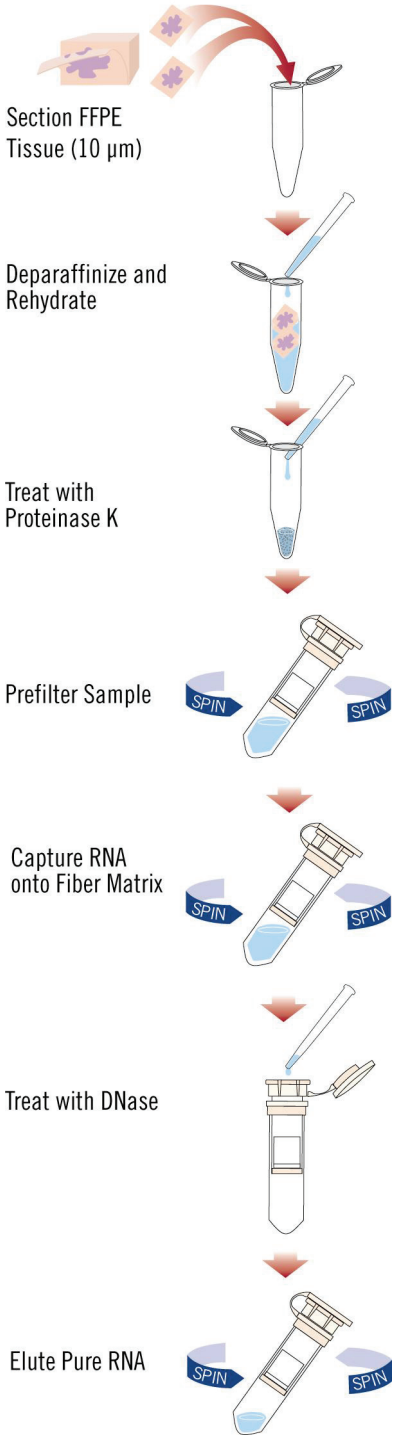

Figure 1 Overview of the Absolutely RNA FFPE kit procedure.

Stratagene QPCR Human Reference Total RNA

The Absolutely RNA FFPE kit includes the Stratagene QPCR Human Reference Total RNA (QPCR Reference RNA) to facilitate QPCR assay optimization, validation, and calibration within and across experiments.

The provided QPCR Reference RNA represents an abundant, high quality template that may be used to optimize QPCR assays for each of the experimental and normalizer targets used in your experiments. This allows you to conserve precious FFPE RNA samples for experiments using optimized assays.

In addition to optimization, the QPCR Reference RNA may be used for assay validation and calibration. QRT-PCR experiments using mRNA from FFPE tissues have a high intrinsic degree of variability, due to differences in tissue fixation and harvesting protocols, different tissue sources and the age and condition of the archived material. When validating QRT-PCR assays for use with FFPE samples, a high-quality control RNA is critical for determination of assay-specific quantitative linearity and dynamic range. Likewise, when performing comparative quantitation analysis on FFPE purified RNA, it is important to use a stable calibrator sample to reduce variance introduced in relative quantity calculations.

Composition of the QPCR Reference RNA

The QPCR Reference RNA is a high-quality control for quantitative PCR gene-expression analysis. The QPCR Reference RNA is composed of total RNA from 10 human cell lines (see the table below), with quantities of RNA from the individual cell lines optimized to maximize representation of gene transcripts present in low, medium, and high abundance. The reference RNA is carefully screened to ensure the absence of contaminating genomic DNA. This reference RNA is also available separately (catalog #750500).

Stratagene QPCR Reference Total RNA, Human Cell Line Derivations
Adenocarcinoma, mammary gland
Hepatoblastoma, liver
Adenocarcinoma, cervix
Embryonal carcinoma, testis
Glioblastoma, brain
Melanoma, skin
Liposarcoma
Histiocytic lymphoma; macrophage; histocyte
Lymphoblastic leukemia, T lymphoblast
Plasmacytoma; myeloma; B lymphocyte

PROTOCOL

Preparing the Reagents

90% Sulfolane

Prepare 90% (v/v) sulfolane by diluting 100% sulfolane with RNase-free water.

Preparation of 12 ml of 90% sulfolane is sufficient for processing 50 RNA preparations. To prepare 12 ml of 90% sulfolane, add 1.2 ml of RNase-free water to 10.8 ml of 100% sulfolane.

Note *100% sulfolane is a solid at room temperature. Prior to diluting the sulfolane, melt by incubating in a 37°C waterbath until liquefied (overnight incubation is convenient for this purpose). The 90% sulfolane solution is a liquid at room temperature, and may be stored at room temperature for at least one month.*

If particulate matter is observed, the sulfolane solution may be filtered using a 0.2 µm nylon filter.

RNase-Free DNase I

Reconstitute the lyophilized RNase-free DNase I by adding 290 µl of DNase Reconstitution Buffer to the vial. Mix the contents thoroughly to ensure that all the powder goes into solution. Do not introduce air bubbles into the solution. Store the reconstituted RNase-Free DNase I at –20°C.

Note *DNase Reconstitution Buffer is easily added to the vial of DNase with a syringe and needle. Gentle mixing is necessary because the DNase I is very sensitive to denaturation.*

High-Salt Wash Buffer

Prepare 1× High-Salt Wash Buffer by adding 16 ml of 100% ethanol to the bottle of 1.67× High-Salt Wash Buffer.

After adding the ethanol, mark the container as suggested: *[√] 1× (Ethanol Added).* Cap the container of 1× High-Salt Wash Buffer tightly and store at room temperature.

Low-Salt Wash Buffer

Prepare 1× Low-Salt Wash Buffer by adding 68 ml of 100% ethanol to the bottle of 5× Low-Salt Wash Buffer.

After adding the ethanol, mark the container as suggested: *[√] 1× (Ethanol Added).* Cap the container of 1× Low-Salt Wash Buffer tightly and store at room temperature.

Ethanol Wash Solutions

Prepare 90% (v/v) and 70% (v/v) ethanol solutions (1 ml of each wash solution per tissue sample) using DNase-, RNase-free H_2O.

Preparing the FFPE Tissue Samples

1. Using a clean, sharp microtome blade, cut two 10-micron thick sections from a trimmed FFPE tissue block (approximately 0.5–1.5 cm^2 surface area per section).

Note *It is good practice to trim the face of the tissue block prior to cutting the samples that will be used for RNA isolation by cutting and discarding several 10-micron thick sections prior to cutting the sample sections.*

2. Place the two sections in a single 1.5-ml microcentrifuge tube and cap the tube.

Note *Do not exceed the recommended amount of tissue. Using excess FFPE tissue may decrease the yield of isolated RNA.*

Sample Deparaffinization and Rehydration

1. Add 1 ml of Deparaffinization Reagent to each tube.

2. Vortex for 10 seconds, and then incubate the tubes at room temperature for 10 minutes. During the 10-minute incubation, vortex the tubes for 10 seconds, twice, at regular intervals.

3. Spin the tubes for 5 minutes at maximum speed (14,000 rpm/ 16,000 × *g*) in a microcentrifuge, and then carefully remove and discard the supernatant using a pipetteman.

   Note *For the remainder of the protocol, pellets will be loose after centrifugation. Use care when removing all solutions.*

4. Repeat steps 1–4 with a fresh 1-ml aliquot of Deparaffinization Reagent.

   Note *For tissue blocks that contain an excess of paraffin, an additional round of treatment with Deparaffinization Reagent may increase RNA yield.*

5. Wash the pellets by adding 1 ml of 100% ethanol. Flick the tubes to dislodge the pellets and then vortex the tubes for 10 seconds.

6. Spin the tubes for 5 minutes at maximum speed, and then carefully remove and discard the supernatant using a pipetteman.

7. Repeat steps 5 and 6 using 90% ethanol.

8. Rehydrate the samples by repeating steps 5 and 6 using 70% ethanol.

9. After removing the 70% ethanol, re-spin the tubes for 1 minute at maximum speed. Remove any residual fluid using a fine-bored pipette tip.

10. Allow the pellets to air dry for 5 minutes at room temperature.

Proteinase K Digestion

1. Prepare a working solution of Proteinase K by combining 100 µl of Proteinase K Digestion Buffer and 10 µl of Proteinase K per sample.

   Note *Prepare a fresh working solution of Proteinase K before each use.*

2. Add 110 µl of the Proteinase K working solution to each sample pellet. Incubate the tubes at 55°C for 3 hours, or until the digestion mixture has clarified (up to 18 hours). The amount of time required for Proteinase K digestion varies for different tissue types and for different sample preparations, but a 3-hour incubation is sufficient for most samples.

   Note *At this point, samples may be stored at –80°C for future processing.*

RNA Isolation

1. For each tissue sample, add 0.875 µl of β-ME to 125 µl of RNA Binding Buffer. (Once opened, store the β-Mercaptoethanol at 4°C.)

   Caution *The RNA Binding Buffer contains the irritant guanidine thiocyanate.*

   Note *Prepare a fresh mixture of RNA Binding Buffer and β-ME before each use.*

2. Add 125 µl of the RNA Binding Buffer–β-ME mixture to each sample and vortex or pipet the sample repeatedly until homogenized.

3. Transfer each sample to a prefilter spin cup. Place the spin cup in the 2-ml receptacle tube provided and spin in a microcentrifuge for 1 minute at maximum speed (14,000 rpm/16,000 × *g*).

4. To each filtrate in the receptacle tubes, add an equal volume of 90% sulfolane (235 µl) and mix thoroughly by vortexing for 5 seconds.

   Note *It is very important to use equal volumes of 90% sulfolane and tissue lysate. It is also important to vortex until the lysate and sulfolane are thoroughly mixed.*

5. Transfer this mixture to a RNA-Binding Spin Cup that has been seated within a 2-ml receptacle tube and snap the cap of the tube onto the top of the spin cup.

6. Spin in a microcentrifuge for 30–60 seconds at 14,000 × *g* to load the RNA onto the spin cup matrix.

   Note *Do not exceed a spin speed of 14,000* × g *while the RNA is bound to the RNA-Binding Spin Cup matrix (steps 6–14).*

7. Remove and **retain the spin cup** and discard the filtrate.

   Note *Up to this point, the RNA has been protected from RNases by the presence of guanidine thiocyanate.*

8. Replace the spin cup in the receptacle tube, then add 600 µl of 1× Low-Salt Wash Buffer and cap the tube. Spin the sample in a microcentrifuge at 14,000 × *g* for 30–60 seconds.

9. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube, cap the tube, and spin the sample in a microcentrifuge at 14,000 × *g* for 2 minutes to dry the fiber matrix.

10. Prepare the DNase treatment solution by gently mixing 5 µl of reconstituted RNase-free DNase I with 25 µl of DNase Digestion Buffer.

    Notes *Gentle mixing is necessary because the DNase I is very sensitive to denaturation.*

    Store the reconstituted DNase I at –20°C.

11. Add the 30 µl of DNase treatment solution directly onto the fiber matrix inside the spin cup and cap the tube. Place the samples in a 37°C incubator for 15 minutes.

12. Add 500 µl of 1× High-Salt Wash Buffer to the spin cup, cap the tube, and spin the sample in a microcentrifuge at 14,000 × *g* for 30–60 seconds.

    Caution *The High-Salt Wash Buffer contains the irritant guanidine thiocyanate.*

13. Remove and **retain the spin cup,** discard the filtrate, and replace the spin cup in the receptacle tube. Add 600 µl of 1× Low-Salt Wash Buffer. Cap the tube and spin the sample in a microcentrifuge at 14,000 × *g* for 30–60 seconds.

14. Remove and **retain the spin cup,** discard the filtrate, and replace the spin cup in the receptacle tube. Add 300 µl of 1× Low-Salt Wash Buffer. Cap the tube and spin the sample in a microcentrifuge at 14,000 × *g* for 30–60 seconds.

15. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube, cap the tube, and spin the sample in a microcentrifuge at 14,000 × *g* for 2 minutes to dry the fiber matrix.

16. Transfer the spin cup to a 1.5-ml collection tube (provided).

17. Pre-heat the Elution Buffer (30 µl per sample) to 75°C. Add 30 µl of Elution Buffer directly onto the fiber matrix inside the spin cup. Snap the cap of the collection tube onto the spin cup and incubate the sample at room temperature for 2 minutes.

    Note *The Elution Buffer must be added directly onto the fiber matrix of the spin cup to ensure that the Elution Buffer permeates the entire fiber matrix.*

18. Spin the sample in a microcentrifuge at maximum speed for 1 minute.

19. The purified RNA is in the Elution Buffer in the microcentrifuge tube. Cap the tube to store the RNA. The RNA can be stored at –20°C for up to one month or at –80°C for long-term storage.

Assessing RNA Quantity and Quality

To quantify RNA, measure the optical density (OD) at 260 nm and calculate the yield, based on the RNA extinction coefficient (1 unit A_{260} = 40 µg/ml). Yield will vary with the tissue type and sample origin. Typically, yields of 0.5–10 µg RNA are obtained from samples derived from two tissue sections with a total surface area of 1–3 cm^2.

Notes *The OD_{260} can be measured conveniently by analyzing a small aliquot of the sample directly using a small-volume spectrophotometer such as a NanoDrop® instrument. If this option is not available, remove a small sample and dilute it with a buffer of neutral pH (e.g., 10 mM Tris, pH 7.5) and measure the OD_{260} using a conventional spectrophotometer.*

For samples that produce lower yields of RNA, a more sensitive fluorescence-based system (e.g., RiboGreen® RNA quantitation kit, Molecular Probes, Inc.) may be used to quantify the RNA.

RNA purity may be assessed by determining the A_{260}/A_{280} ratio. The expected ratio is approximately 1.8–2.2.

To qualify the RNA based on the size of recovered RNA molecules, the sample may be assessed by agarose gel electrophoresis or by microfluidics analysis, e.g., using the Agilent 2100 Bioanalyzer with an RNA LabChip® kit. Using either method, the RNA recovered from FFPE samples is expected to be smaller and more heterogeneous (i.e. bands or peaks are smeared) compared to RNA from equivalent fresh or frozen tissues. The 18S and 28S rRNA bands or peaks will likely be broadened and diminished or may be absent altogether. There is wide variation in the average size of RNAs from FFPE tissues, with older tissue samples typically producing a shorter average RNA size.[7] However, even highly degraded RNA samples (with an average size of ~100 nucleotides) are typically suitable for QRT-PCR analysis, as long as short amplicons are used for PCR detection (≤100 bp amplicons). See Figure 2 for a representative Agilent Bioanalyzer profile of RNA size distribution for total RNA recovered from FFPE tissues.

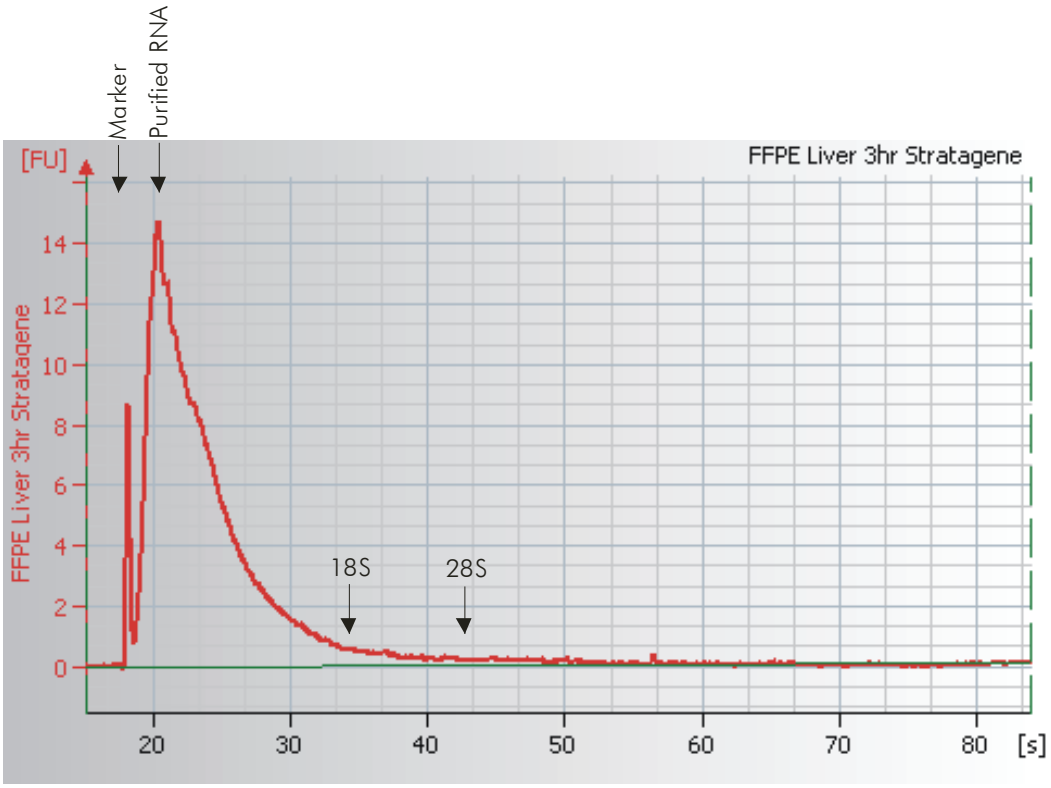

Figure 2 Electropherogram showing a typical size distribution of FFPE RNA. Total RNA was isolated from two 10-micron sections of human liver FFPE tissue (stored for 8 years) using the Absolutely RNA FFPE kit and analyzed using the Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip kit. RNA yield was 10.4 µg. As a result of degradation, the majority of RNA was between 100–300 bases in length and 18S and 28S rRNA peaks were not observed.

RNA ANALYSIS BY QRT-PCR

QRT-PCR Assay Design Considerations

Amplicon size

Because of the small size of the RNAs isolated from FFPE tissues, it is important to design QRT-PCR assays using an amplicon size ≤100 bp. For accurate normalization, the sizes of the amplicons used to detect the target gene and all reference genes should be as similar as possible.[7]

Detection Method

Either probe-based or SYBR® Green dye-based detection methods may be used for the QRT-PCR assays. Keep in mind, however, that the use of short amplicons (≤100 bp) is critical. SYBR Green dye-based assays, which typically employ longer amplicon sizes, may need to be re-optimized with new primer sets.

cDNA Synthesis Primers

Use either gene-specific or random primers for cDNA synthesis. Since most of the mRNA molecules isolated from FFPE tissues do not contain a poly(dA) sequence, oligo(dT) primers should not be used.

Cycle Number

Performing QRT-PCR with RNA isolated from FFPE samples typically produces threshold cycle (Ct) values that are several cycles higher than the Ct values produced from the same mass units of RNA isolated from the equivalent fresh or frozen tissue. The cycle number should therefore be increased by 5–10 cycles.

Guidelines for Using the Stratagene QPCR Human Reference Total RNA

Assay Optimization and Efficiency Determinations

Before initiating RT-PCR analysis using the recovered FFPE RNA as template, ensure that the RT-PCR assay is optimized for the target using the specific primers and probe selected for analysis. If assay optimization is required, use the provided Stratagene QPCR Human Reference Total RNA (QPCR Reference RNA) as template during optimization.

Once the optimal primer and probe concentrations have been determined, it is advisable to assess the linearity and sensitivity of the assay by generating a standard curve using serial dilutions of the QPCR Reference RNA. See *Generating a QPCR Reference RNA Standard Curve*, below, for guidelines. Standard curves should be performed for each of the targets used in the experiment (gene of interest as well as all normalizer targets) to determine reliable dynamic ranges and accurate efficiencies to be used in data anlysis.

Generating a QPCR Reference RNA Standard Curve

To generate a standard curve, we suggest including the following amounts of the QPCR Reference RNA in 25-µl QRT-PCR reactions, according to the abundance of the specific target. The amount of template may need to be adjusted for certain probe/primer systems.

Target mRNA abundance	Suggested fold dilution	Final amounts RNA template (ng) in standard curve reactions (25 µl)
High	10-fold	100, 10, 1, 0.1, 0.01
Medium	5-fold	100, 20, 4, 0.8, 0.16
Low	2-fold	100, 50, 25, 12.5, 6.25

Prepare the appropriate dilutions of the QPCR Reference RNA in RNase-free H_2O. It is typically convenient to prepare an initial 40 ng/µl dilution of the reference RNA stock, and then prepare serial dilutions from this solution, using the fold-dilution scheme shown in the table above. Using this method, 2.5 µl of each serial dilution may be added to the 25-µl reactions to achieve the desired amounts of QPCR Reference RNA template in each reaction. Standard curve reactions should be performed in triplicate.

Use as an Exogenous Calibrator in Comparative Quantitation Experiments

Experiments for comparing gene expression levels in different tissue samples are often based on comparative quantitation (also referred to as relative quantitation) QRT-PCR methods. When performing comparative quantitation experiments, results are expressed as the fold difference between the target gene expression in experimental samples versus a calibrator sample. The QPCR Reference RNA can be used as an exogenous calibrator sample in these experiments. Using this approach, Ct values are collected for the gene of interest in the calibrator sample (a single amount of reference RNA) and in experimental (unknown) samples in the same run. For each experimental sample, the fold-difference in Ct value relative to the reference RNA calibrator is expressed as the relative quantity. These experiments typically also include analysis of a normalizer target (a gene whose expression is expected to be constant in all samples) in both calibrator and experimental samples. This normalization controls for differences in RNA isolation and in the efficiency of reverse transcription arising sample to sample and experiment to experiment. The normalized relative quantities of the gene of interest in different experimental samples may then be compared to each other, as each relative quantity value is made in comparison to the same benchmark sample.

Cross-Experiment Comparison Applications

QRT-PCR experiments are generally associated with run-to-run variation, and experiments designed to analyze RNA from FFPE tissues have an especially high intrinsic degree of variability due to differences arising from the source material.

For these reasons, some researchers include a standard curve in each experimental run, using an abundant template source such as the QPCR Reference RNA. (See *Generating a QPCR Reference RNA Standard Curve*, above, for guidelines.) Including a QPCR Reference RNA standard curve in each experiment provides a data set that may be used for normalization of results obtained in different experiments. One way of analyzing these data is utilizing the calculation of the Confidence Interval (CI) for the data points of the standard curve. Reference 8 provides a useful discussion of the calculation and use of confidence interval values in comparing data sets.

TROUBLESHOOTING

Observation	Suggestion
RNA is degraded	Degradation is expected for RNAs isolated from FFPE tissues, and different tissue samples vary widely in the average RNA size. Older tissue samples typically contain smaller RNA molecules.
	Proteinase K digestion should be carried out at 55°C. Subjecting the RNA to higher temperatures can increase RNA degradation.
	Ensure that the standard handling precautions for RNA are used after RNA isolation. See Appendix II: *Preventing Sample Contamination* for more information.
RNA yield is poor	Confirm that the tissue section size is within the recommended range of 1–3 cm^2 surface area of 10-µm sections. Using excess starting material can reduce RNA yield.
	Confirm that the 90% sulfolane and the pre-filter spin cup filtrate were combined at a 1:1 ratio prior to loading the RNA-binding spin cup.
	The amount of RNA that can be isolated from different tissue types varies widely.
	If the FFPE tissue block appears to contain excess paraffin, including an additional round of deparaffinization (for a total of three rounds) may increase RNA yield.
	The proteinase K digestion of the sample may be incomplete. Perform the proteinase K digestion for up to 18 hours, until the digestion suspension appears clarified.
	Incubate the spin cup for two full minutes after adding the Elution Buffer.
Final RNA concentration is too low for use in subsequent applications	Concentrate the RNA under vacuum without heat.
	Use a smaller volume of Elution Buffer, ensuring that the surface of the fiber matrix is completely covered. (It is possible to use as little as 10 µl of Elution Buffer as long as the fiber matrix is completely covered. The use of <30 µl of Elution Buffer, however, will lower the RNA yield.)
DNA contamination, suggested by DNA amplification in no-RT controls	Ensure that the spin cup is centrifuged at maximum speed for 2 full minutes before DNase treatment.
	DNase I is highly sensitive to denaturation. Ensure that the DNase I solution is prepared and stored according to the instructions in the *Protocol* section.
No products detected in QRT-PCR assays	Ensure that the QRT-PCR assay uses short amplicons (≤100 bp).
	Optimize the QRT-PCR assay, using the QPCR Reference RNA as template.
	Verify that either gene-specific or random primers were used for first-strand synthesis. Do not use oligo(dT) primer.
	Ensure that a sufficient cycle number is used in the amplification protocol. RNA isolated from FFPE tissues typically displays a Ct that is a few cycles higher than RNA obtained from other sources.

APPENDIX I: ISOLATING RNA FROM SLIDE-MOUNTED FFPE TISSUE

This protocol can be used to isolate the RNA from FFPE tissue sections that have been mounted on microscope slides. It is most convenient to process several slides simultaneously, using a slide rack plus a series of glass staining dishes. The protocol may also be adapted for a single slide, using a smaller container.

This protocol requires excess Deparaffinization Reagent (d-limonene). A suitable replacement d-limonene solution (Hemo-De, catalog #HD150A) is available from Scientific Safety Solvents (Keller, TX). The protocol requires approximately 100–200 ml of d-limonene solution per rack of slides or 1–10 ml of d-limonene solution per single slide (depending on the containers used).

Sample Deparaffinization and Rehydration

1. Place slides in a slide rack, then place the rack in a clean staining dish containing a sufficient volume of d-limonene to cover the slide-mounted sections. Incubate the slides at room temperature for 15–20 minutes.

2. Remove the slide rack from the dish, blot the excess d-limonene, and transfer the rack to a second staining dish containing 100% ethanol (sufficient volume to cover the samples). Incubate the slides at room temperature for 10 minutes.

3. Transfer the rack to a third staining dish containing 90% ethanol (sufficient volume to cover the samples). Incubate the slides at room temperature for 10 minutes.

4. Transfer the rack to a fourth staining dish containing 70% ethanol (sufficient volume to cover the samples). Incubate the slides at room temperature for 15 minutes.

5. Using a sterile, single-use scalpel, scrape the deparaffinized tissue section from each slide into a separate 1.5 ml tube containing 0.5 ml of 70% ethanol.

   Note *If desired, two sections from the same tissue block may be combined in the same tube.*

6. Spin the tubes for 5 minutes at maximum speed, and then carefully remove and discard the supernatant using a pipetteman.

7. Re-spin the tubes for 1 minute at maximum speed. Remove any residual fluid using a fine-bored pipette tip.

8. Allow the pellets to air dry for 5 minutes at room temperature.

9. Proceed to *Proteinase K Digestion* in the main *Protocol* section of this manual. Complete the remaining steps, including Proteinase K digestion, RNA isolation, and quantification as described.

APPENDIX II: PREVENTING SAMPLE CONTAMINATION

Preventing RNase Contamination

Ribonucleases are very stable enzymes that hydrolyze RNA. RNase A can be temporarily denatured under extreme conditions, but it readily renatures. RNase A can therefore survive autoclaving and other standard methods of protein inactivation. The following precautions can prevent RNase contamination:

- **Wear gloves at all times** during the procedures and while handling materials and equipment**,** as RNases are present in the oils of the skin.

- Exercise care to ensure that all equipment (e.g., centrifuge tubes, etc.) is as free as possible from contaminating RNases. Avoid using equipment or areas that have been exposed to RNases. Use sterile tubes and micropipet tips only.

- Micropipettor bores can be a source of RNase contamination, since material accidentally drawn into the pipet or produced by gasket abrasion can fall into RNA solutions during pipetting. Clean micropipettors according to the manufacturer's recommendations. Rinse both the interior and exterior of the micropipet shaft with ethanol or methanol.

Sterilizing Labware

Disposable Plasticware

Disposable sterile plasticware is generally free of RNases. If disposable sterile plasticware is unavailable, components such as microcentrifuge tubes can be sterilized and treated with diethylpyrocarbonate (DEPC), which chemically modifies and inactivates enzymes, according to the following protocol:

Caution *DEPC is toxic and extremely reactive. Always use DEPC in a fume hood.* ***Read and follow the manufacturer's safety instructions.***

1. Add DEPC to deionized water to a final DEPC concentration of 0.1% (v/v) and mix thoroughly.

2. Place the plasticware to be treated into a separate autoclavable container. Carefully pour the DEPC-treated water into the container until the plasticware is submerged.

3. Leave the container and the beaker used to prepare DEPC-treated water in a fume hood overnight.

4. For disposal, pour the DEPC-treated water from the plasticware into another container with a lid. Autoclave the bottle of waste DEPC-treated water and the container with the plasticware for at least 30 minutes. Aluminum foil may be used to cover the container, but it should be handled with gloves and cut from an area untouched by ungloved hands.

Nondisposable Plasticware

Remove RNases from nondisposable plasticware with a chloroform rinse. Before using the plasticware, allow the chloroform to evaporate in a hood or rinse the plasticware with DEPC-treated water.

Electrophoresis Gel Boxes

To inactivate RNases on electrophoresis gel boxes, treat the gel boxes with 3% (v/v) hydrogen peroxide for 10–15 minutes and then rinse them with RNase-free water.

Glassware or Metal

To inactivate RNases on glassware or metal, bake the glassware or metal for a minimum of 8 hours at 180°C.

Treating Solutions with DEPC

Treat water and solutions (except those containing Tris base) with DEPC, using 0.1% (v/v) DEPC in distilled water. During preparation, mix the 0.1% solution thoroughly, allow it to incubate overnight at room temperature, and then autoclave it prior to use. If a solution contains Tris base, prepare the solution with autoclaved DEPC-treated water.

Preventing Nucleic Acid Contamination

If the isolated RNA will be used for cDNA synthesis for cDNA library construction or PCR amplification, it is important to remove any residual nucleic acids from equipment that was used for previous nucleic acid isolations.

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ENDNOTES

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.

STRATAGENE

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QUICK-REFERENCE PROTOCOL: ABSOLUTELY RNA FFPE KIT

Catalog #400809, 400811

Note *Before starting, ensure that reagents have been prepared as described in the* Preparing the Reagents *section of the main manual.*

Tissue Deparaffinization and Rehydration

1. Cut two 10-micron thick sections (0.5–1.5 cm^2 surface area per section) from a trimmed FFPE tissue block and place the sections in a single 1.5-ml microcentrifuge tube.

2. Add 1 ml of Deparaffinization Reagent to each tube. Vortex for 10 seconds, and then incubate the tubes at room temperature for 10 minutes, vortexing for 10 seconds twice during the incubation.

3. Spin the tubes for 5 minutes at maximum speed in a microcentrifuge, and then carefully remove and discard the supernatant using a pipetteman.

4. Repeat steps 2 and 3 with a fresh 1-ml aliquot of Deparaffinization Reagent.

5. Wash the pellets by adding 1 ml of 100% ethanol. Flick the tubes to dislodge the pellets and then vortex the tubes for 10 seconds. Spin the tubes for 5 minutes at maximum speed, and then carefully remove and discard the supernatant using a pipetteman.

6. Repeat step 5 using 90% ethanol.

7. Repeat step 5 using 70% ethanol.

8. After removing the 70% ethanol, re-spin the tubes for 1 minute at maximum speed. Remove any residual fluid and then allow the pellets to air dry for 5 minutes.

Proteinase K Digestion

9. Prepare a fresh working solution of Proteinase K by combining 100 µl of Proteinase K Digestion Buffer and 10 µl of Proteinase K per sample.

10. Add 110 µl of the Proteinase K working solution to each sample pellet. Incubate the tubes at 55°C for 3 hours, or until the digestion mixture has clarified (up to 18 hours).

RNA Isolation

11. Prepare a fresh working solution of RNA Binding Buffer by combining 125 µl of RNA Binding Buffer and 0.875 µl of β-ME per sample.

12. Add 125 µl of the RNA Binding Buffer–β-ME mixture to each sample and vortex or pipet repeatedly until homogenized.

13. Transfer each sample to a prefilter spin cup. Place the spin cup in a 2-ml receptacle tube (provided) and spin in a microcentrifuge for 1 minute at maximum speed.

14. To the filtrate collected in each tube, add 235 µl of 90% sulfolane. Vortex for 5 seconds.

15. Transfer this mixture to a RNA-Binding Spin Cup that has been seated within a 2-ml receptacle tube (provided) and snap the cap of the tube onto the top of the spin cup. Spin in a microcentrifuge for 30–60 seconds **at 14,000 × *g***.

16. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube, then add 600 µl of 1× Low-Salt Wash Buffer and cap the tube. Spin in a microcentrifuge for 30–60 seconds **at 14,000 × *g***.

17. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube, cap the tube, and spin in a microcentrifuge for 2 minutes **at 14,000 × *g*** to dry the fiber matrix.

18. Prepare the DNase treatment solution by gently mixing 5 µl of reconstituted RNase-free DNase I with 25 µl of DNase Digestion Buffer per sample. Add 30 µl of DNase treatment solution directly onto the fiber matrix inside the spin cup and cap the tube. Incubate the samples at 37°C for 15 minutes.

19. Add 500 µl of 1× High-Salt Wash Buffer and cap the tube. Spin in a microcentrifuge for 30–60 seconds **at 14,000 × *g***.

20. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube, then add 600 µl of 1× Low-Salt Wash Buffer and cap the tube. Spin in a microcentrifuge for 30–60 seconds **at 14,000 × *g***.

21. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube, then add 300 µl of 1× Low-Salt Wash Buffer and cap the tube. Spin in a microcentrifuge for 30–60 seconds **at 14,000 × *g***.

22. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube, cap the tube, and spin in a microcentrifuge for 2 minutes **at 14,000 × *g*** to dry the fiber matrix.

23. Transfer the spin cup to a 1.5-ml collection tube (provided). Add 30 µl of Elution Buffer (pre-heated to 75°C) directly onto the fiber matrix inside the spin cup. Cap the spin cup and incubate the sample at room temperature for 2 minutes.

24. Spin the sample in a microcentrifuge at maximum speed for 1 minute.

25. The purified RNA is in the Elution Buffer in the microcentrifuge tube. Cap the tube and store the RNA at –20°C for up to one month or at –80°C for long-term storage.