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Not for use in diagnostic procedures.

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High Pure FFPET RNA Isolation Kit

Version 03

Content version: October 2012

For the isolation of total RNA from formalin-fixed, paraffin-embedded tissue

Cat. No. 06 650 775 001	**Kit for 50 Isolations**
Cat. No. 06 868 517 001	**Kit for 10 Isolations**

Store the kit at +15 to +25°C

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Table of Contents

1. What this Product Does

Number of Tests

The High Pure FFPET RNA Isolation Kit is available in 2 pack sizes:

Cat. No.	Pack Size
06 868 517 001	10 Isolations
06 650 775 001	50 Isolations

Both sizes of the kit contain the same components; only the amount (values in parentheses below) of the components in the kit changes.

Kit Contents

⚠ All solutions are clear and should not be used when precipitates have formed. Warm the solutions at +15 to +25°C, or in a +37°C water bath, until the precipitates have dissolved.

Vial/cap	Label	Contents/Function
1 red	RNA Tissue Lysis Buffer	▪ 6 ml ▪ For the lysis of cells
2 pink	Proteinase K, PCR grade	▪ Lyophilizate ▪ 100 mg (2 × 100 mg) ▪ For sample homogenization and inactivation of endogenous nucleases
3 green	RNA Binding Buffer	▪ 20 ml ▪ Contains 5 M guanidine thiocyanate ▪ *Store protected from light*
4 black	Wash Buffer I	▪ 25 ml, add 15 ml absolute ethanol ▪ Contains 5 M guanidine HCl (final concentration after addition of ethanol)
5 blue	Wash Buffer II	20 ml, add 80 ml absolute ethanol
6 white	DNase I	▪ Lyophilizate ▪ 100 mg ▪ For digestion of residual DNA ▪ 4 kU DNase I
7 white	DNase Incubation Buffer (1×)	6 ml
8 colorless	RNA Elution Buffer	▪ 1,000 µl (5 × 1,000 µl) ▪ Water, PCR Grade
9 colorless	Reagent Preparation Buffer	11.5 ml
10	High Pure Filter Tubes	10 (50) polypropylene tubes with two layers of glass fiber fleece
11	Collection Tubes	One bag (3 bags) containing 50 polypropylene tubes (2 ml)

Storage and Stability

⚠ The High Pure FFPET RNA Isolation Kit components must be stored at +15 to +25°C. If properly stored, all kit components are stable until the expiration date printed on the label.

⚠ Improper storage at +2 to +8°C (refrigerator) or -15 to -25°C (freezer) will adversely impact nucleic acid purification due to the formation of precipitates in the solutions.

Therefore, the High Pure FFPET RNA Isolation Kit is always shipped at ambient temperature.

After dissolving DNase I lyophilizate and Proteinase K lyophilizate in Reagent Preparation Buffer, aliquot and store the solutions at -15 to -25°C; the solutions are stable for 12 months.

Additional Equipment and Reagents Required

- Pipette tips, PCR grade
- Pipettes
- Vortex
- Thermomixer
- Polypropylene tubes, 1.5 ml, sterile
- Standard tabletop microcentrifuge capable of 16,000 × *g* centrifugal force
- Xylene
- Ethanol absolute
- SDS, 10% (ready-to-use)
- Water, PCR Grade

Application

The High Pure FFPET RNA Isolation Kit uses a fast and optimized technology for the isolation and purification of total RNA from formalin-fixed, paraffin-embedded tissue research samples. The quality of RNA from tissue samples is suitable for the following downstream applications:

- RT-PCR
- Gene Expression Array
- Next Generation Sequencing
- PreAmplification

Assay time

Total time required is approximately 2.5 hours including deparaffinization procedure.

2. How to Use this Product

2.1 Before you begin

Precautions

⚠ Guanidine thiocyanate in RNA Binding Buffer and Guanidine hydrochloride in Wash Buffer I is an irritant. Always wear gloves and follow standard safety precautions to minimize contact when handling.

⚠ Do not allow the RNA Binding Buffer and/or the Wash Buffer I to mix with sodium hypochlorite found in commercial bleach solutions. This mixture can produce a highly toxic gas.

- Do not let these buffers touch your skin, eyes, or mucous membranes. If contact does occur, wash the affected area immediately with large amounts of water; otherwise, the reagent may cause burns. If you spill the reagent, dilute the spill with water before wiping it up.
- Never store or use the RNA Binding Buffer and Wash Buffer I near human or animal food. Store the Binding Buffer protected from light.
- Always wear gloves and follow standard safety precautions when handling these buffers.

Sample Material

- Maximum 10 µm thick sections from formalin-fixed, paraffin-embedded tissue, such as colon, breast, lung, and kidney from mammalian species including human research samples.
- Macrodissected samples (typically 3 - 5 µm) or biopsy material, such as colon, breast, lung, and kidney from mammalian species including human research samples.
- Laser Microdissection (LMD) samples (typically 3 - 5 µm), for example lung and breast tissue from mammalian species including human research samples. (minimum 1 mm^2, 25 µl elution volume is recommended for microdissected sample material).

Handling Instructions

During fixation in formalin, intracellular RNases become inactivated. However, RNA is degraded and crosslinked to proteins inter- and intra-molecularly. For this reason, formalin-fixed, paraffin-embedded tissue can be stored and handled at +15 to +25°C.

⚠ It is recommended to use sterile disposable polypropylene tubes and tips. Always wear gloves during the assay.

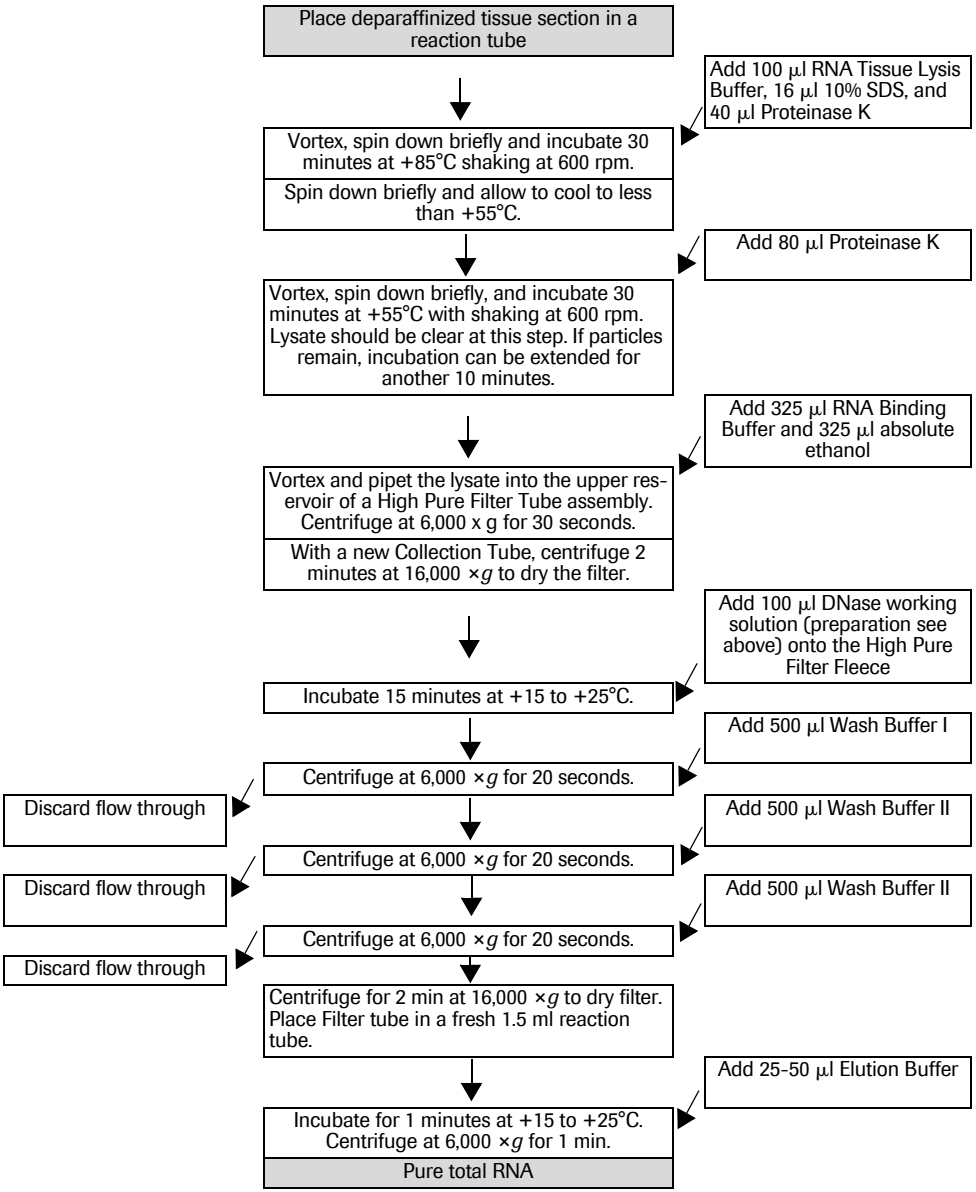
Preparation of Working Solutions

Besides the ready-to-use solutions supplied with this kit, you will need to prepare the following working solutions.

Content	Reconstitution/ Preparation	Storage and Stability	For use in
Wash Buffer I (Vial 4, black cap)	Add 15 ml absolute ethanol.	Store prepared Wash Buffer I at +15 to +25°C.	RNA Isolation Protocol, washing and elution Step 7
	⚠ Label and date vial accordingly after addition of ethanol.		
Wash Buffer II (Vial 5, blue cap)	Add 80 ml absolute ethanol.	Store prepared Wash Buffer II at +15 to +25°C.	RNA Isolation protocol, washing and elution Steps 8 and 9
	⚠ Label and date vial accordingly after addition of ethanol.		
Proteinase K (Vial 2, pink cap)	Dissolve contents of Bottle 2 in 4.5 ml Reagent Preparation Buffer (Vial 9).	Prepare aliquots according to the expected number of samples. Label and store aliquots at -15 to -25°C.	RNA Isolation Protocol, sample lysis Step 1 and 2
DNase I (Vial 6, white cap)	Dissolve contents of Bottle 6 in 740 µl Reagent Preparation Buffer (vial 9).	Prepare aliquots according to the expected number of samples. Label and store aliquots at -15 to -25°C.	Preparation of DNase working solution
DNase working solution	Prepare DNase working solution (see note below)	Prepare required amount for expected number of samples. DNase working solution cannot be stored and should be prepared for each extraction round.	RNA Isolation Protocol, washing and elution Step 6

Required amount for N (number of) RNA isolations.
(N+1) × 90 µl DNase Incubation Buffer (Vial 7, white) + (N+1) × 10 µl DNase I (dissolved, see above).

2.2 Experimental Overview



2.3 Protocol for the Isolation or RNA from Formalin-Fixed, Paraffin-Embedded Tissue

Deparaffinization

The following steps describe the deparaffinization procedure for a single maximum 10 µm thick section of formalin-fixed, paraffin-embedded tissue in a 1.5 ml reaction tube.

❶	To one maximum 10 µm section in a 1.5 ml reaction tube, add 800 µl xylene, and vortex briefly in several intervals.
❷	Add 400 µl absolute ethanol and vortex briefly. Centrifuge for 2 minutes at maximum speed (16,000 ×*g*) and discard supernatant. Be careful not to dislodge the pellet.
❸	Add 1 ml absolute ethanol and vortex briefly. Centrifuge for 2 minutes at maximum speed (16,000 ×*g*) and discard supernatant. Be careful not to dislodge the pellet.
❹	Blot the tube briefly onto a paper towel to get rid of ethanol residues. Dry the tissue pellet for 10 minutes at +55°C (open tubes). If required, dry for up to a maximum of 20 minutes. Proceed to Step 1 of the RNA isolation protocol.

Alternative Procedure for Macro-dissection

The following steps describe the deparaffinization procedure for a single section of formalin-fixed, paraffin-embedded tissue on a microscope slide (typically 3-5 µm).

❶	Place the slide in a xylene bath and incubate for 2 minutes.
❷	Change bath and incubate the slide for an additional 2 minutes in xylene.
❸	Tap off excess liquid and place the slide into ethanol abs. for 1 minute.
❹	Change bath and incubate the slide for 1 minute in ethanol 96%.
❺	Change bath and incubate the slide for 1 minute in ethanol 70%.
❻	Change bath and incubate the slide for 1 minute in H_2O (Water PCR Grade).
❼	Add 5-10 µl RNA Tissue Lysis Buffer (Vial 1, red cap) on top of the section. ⚠ Volume needs to be subtracted from amount of RNA Tissue Lysis Buffer in Step 1, RNA Isolation Protocol.

❽	Remove the deparaffinized section from the slide by using a sterile single-use scalpel and place it into a 1.5 ml reaction tube. ⚠ To avoid scattering of the tissue, remove the section from the microscope slide before it has dried. Proceed to Step 1 of the RNA isolation protocol.

Alternative Procedure for Laser Microdissections (LMD)

The following steps describe the deparaffinization procedure for a single section of formalin-fixed, paraffin-embedded tissue on a microscope slide suitable for microdissection (typically 3 - 5 μm).

⚠ Depending on the type of laser capture microscope, special slides may have to be used (*e.g.,* membrane slides).

❶	Place the slide in a xylene bath and incubate for 2 minutes.
❷	Change bath and incubate the slide for an additional 2 minutes in xylene.
❸	Tap off excess liquid and place the slide into ethanol abs. for 1 minute.
❹	Change bath and incubate the slide for 1 minute in ethanol 96%.
❺	Change bath and incubate the slide for 1 minute in ethanol 70%.
❻	Change bath and incubate the slide for 1 minute in H_2O (Water PCR Grade).
❼	Dry the tissue on the slide for approximately 1 hour at +15 to +25°C.
❽	Microdissect your sample material according to the manufacturers' instructions. It is recommended to collect your microdissected material in liquid. You can use RNA Tissue Lysis Buffer (Vial 1, red cap). ⚠ Volume needs to be subtracted from amount of RNA Tissue Lysis Buffer in Step 1, RNA Isolation Protocol.
❾	After capturing the sample material, proceed with Step 1 of the RNA isolation protocol. ⏸ Optionally: Samples can be frozen at - 15 to - 25°C until further processing.

⚠ For RT-qPCR analysis, approximately 1-2 mm^2 of laser microdissected material is required if you are not using an additional pre-amplification step.

RNA Isolation Protocol

The following protocol describes the RNA isolation from a single maximum 10 µm thick section of formalin-fixed, paraffin-embedded tissue or a macro-/microdissected tissue sample (typically 3 - 5 µm).

Depending on the size of the embedded tissue, the number of preparations could be pooled in Step 4:

- For 10 µm sections: 2
- For macrodissected samples: 3
- For LMD: >3 preparations

1	▪ To one tissue pellet (deparaffinized as described above), add 100 µl RNA Tissue Lysis Buffer (Vial 1, red cap), 16 µl 10% SDS, and 40 µl Proteinase K working solution (thawed aliquot). Reagents can be added separatly or as a mix. ▪ Vortex several seconds. ▪ Spin down briefly. ▪ Incubate 30 minutes at +85°C, with shaking at 600 rpm. ▪ Spin down briefly. ▪ Cool to less than +55°C before adding Proteinase K.
2	▪ Add 80 µl Proteinase K (from thawed aliquot; discard unused enzyme). ▪ Vortex several seconds. ▪ Spin down briefly. ▪ Incubate 30 minutes at +55°C, while shaking at 600 rpm. ▪ Spin down briefly. The lysate should be clear at this step. If particles remain, incubation can be extended for another 10 minutes.
3	▪ Add to tissue lysate: 325 µl RNA Binding Buffer (Vial 3, green cap) 325 µl absolute ethanol ▪ Vortex several seconds. ▪ Spin down briefly.
4	▪ Place High Pure Filter Tube onto High Pure Collection Tube. ▪ Pipet lysate into the upper reservoir of the High Pure Filter Tube (volume is approximately 900 µl). ▪ Centrifuge 30 seconds at 6,000 ×*g*.
5	▪ Place High Pure Filter Tube onto a new High Pure Collection Tube. ▪ Centrifuge 2 minutes at 16,000 ×*g* to dry filter fleece completely.
6	▪ Place High Pure Filter Tube onto new High Pure Collection Tube. ▪ Add 100 µl DNase working solution (preparation see above) onto the High Pure Filter Tube fleece without touching the fleece. ▪ Incubate 15 minutes at +15 to +25°C.

❼	▪ Add 500 µl Wash Buffer I working solution (Vial 4, black cap). ▪ Centrifuge 20 seconds at 6,000 ×*g*. Discard flow through.
❽	▪ Add 500 µl Wash buffer II working solution (Vial 5, blue cap). ▪ Centrifuge 20 seconds at 6,000 ×*g*. Discard flow through
❾	▪ Add 500 µl Wash buffer II working solution (Vial 5, blue cap). ▪ Centrifuge 20 seconds at 6,000 ×*g*. Discard flow through. ▪ Centrifuge for 2 minutes at 16,000 ×*g* to dry filter fleece completely. ▪ Place the High Pure Filter Tube into a fresh 1.5 ml reaction tube.
❿	▪ Add 25-50 µl RNA Elution Buffer (Vial 8, colorless cap) to the center of the fleece tube without touching the fleece. ▪ Incubate 1 minute at +15 to +25°C. ▪ Centrifuge for 1 minute at 6,000 ×*g*. A standard elution volume of 50 µl can be applied. If you are working with small amounts of sample material and low RNA yields are expected, it is recommended to reduce the elution volume to 25 µl.
⓫	The microcentrifuge tube now contains the eluted RNA. Use eluted RNA directly or store at + 2 to + 8°C (short term) or –60 to –80°C (long term). ⚠ Before photometric determination or capillary gel electrophoresis of the RNA, centrifuge the eluate for 2 minutes at maximum speed, and transfer supernatant to a fresh 1.5 ml reaction tube without disturbing glass fibers at the bottom of the original tube.

3. Results

Purity

Purified RNA is free of DNA, nucleases, and all cellular and sample components that interfere with RT-PCR, according to the current Quality Control Procedures.

The purity is determined by calculation of OD $_{260/280\ nm}$.

Ratio $_{260/280}$ Eluate: 1.8 - 2.0

RNA Integrity and size distribution

The size of the isolated RNA fragments is analyzed using the Agilent Bioanalyzer. Ninety percent of RNA fragments are greater than 100 nucleotides (exemplary data). The RIN number is usually very low for FFPET-derived RNA samples due to fragmentation. RIN should be >1.4 as a general rule, but even RNA samples with no RIN may work if your PCR amplicons are small.

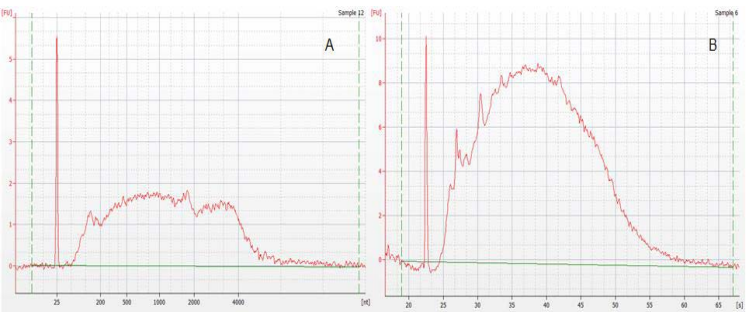


Figure 1: Electropherograms obtained from FFPET-derived RNA generated by the Agilent 2100 Bioanalyzer.

A: The trace shows the result of a purification from a Xenograft (KPL4, BrCa) 5 µm section in an elution volume of 25 µl. Corresponding RIN is 2.4, OD_{260nm} measurement shows a concentration of 77 ng/µl.

B: The trace shows the results of a purification from 2 NSCLC (Non-small-cell lung carcinoma) 10 µm sections (pooled in step 4 of the RNA Isolation Protocol) in an elution volume of 50 µl. Corresponding RIN is 2.2, OD_{260nm} measurement shows a concentration of 275 ng/µl.

RNA yield and integrity strongly depends on sample type, quality, amount, fixation conditions, and time of storage.

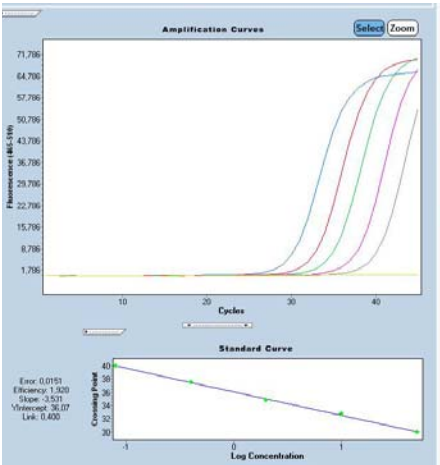


Figure 2: Typical results for a RT-qPCR experiment.
cDNA synthesis has been performed using the Transcriptor Universal cDNA Master. The expression level of the reference gene ALAS 1 (assayID 102108) was determined in a serial dilution of RNA (50 ng to 0.08 ng/PCR) isolated from NSCLC (Non-small-cell lung carcinoma) samples.

4. Troubleshooting

Problem	Cause	Recommendation
Low RNA yield or purity.	Kit stored under non-optimal conditions.	Store kit at +15 to +25°C at all times after arrival.
	Buffers or other reagents were exposed to conditions that reduced their effectiveness.	Store all buffers at +15 to +25°C. Close all reagent vials tightly after each use to preserve pH, stability, and freedom from contamination. After reconstitution of all lyophilizates, aliquot and store at –15 to –25°C
	Precipitates in the Tissue Lysis Buffer, SDS, or Proteinase K.	Do not work on ice as SDS precipitates below room temperature (+15 to +25°C).
	Precipitates in the SDS stock solution.	SDS precipitates below room temperature, therefore dissolve precipitates in a water bath at +37°C.
	Ethanol not added to Wash Buffer.	Add absolute ethanol to buffers before using. After adding ethanol, mix buffers well and store at +15 to +25°C. Always mark buffer vials containing added ethanol.
	Reagents and samples not completely mixed	Always mix the sample tube well after addition of each reagent.
	Ethanol not added to the lysate in Step 3.	Addition of 0.5 volume of absolute ethanol to the lysate is necessary to promote selective binding of RNA to the glass fibers.
	High level of RNase activity	Be careful to create an RNase-free working environment. Process starting material immediately or store at –60 to –80°C until it can be processed. Use eluted RNA directly in downstream procedures or store immediately at –60 to –80°C.
	RNA concentration too low for your application.	Reduce the elution volume to a minimum of 25 µl.

Problem	Cause	Recommendation
$OD_{260\ nm}$ value of eluate too high.	Glass fibers, which might coelute with nucleic acids, scatter light.	1. Remove High Pure Filter Tube from tube containing the eluted sample and centrifuge sample tube for 1 minutes at maximum speed. 2. Transfer supernatant into a new tube without disturbing the glass fibers at the bottom of the original tube.
Samples "pop" out of wells in agarose gels.	Eluate contains ethanol (from the Wash Buffer).	1. After the wash step, do not let the flow through touch the bottom of the High Pure Filter Tube. 2. Empty collection tube, reinsert filter tube in emptied collection tube, and recentrifuge for 30 seconds.

5. Additional Information on this Product

How this Product Works

FFPE tissue samples are disrupted and homogenized during incubation with RNA Tissue Lysis Buffer and Proteinase K. Nucleic Acids (NA) bind in the presence of a chaotropic salt specifically to the surface of glass fibers pre-packed in the High Pure Purification Filter Tube.

RNA is purified in a series of rapid "wash-and-spin" steps to remove salts, proteins, and cellular components. Residual DNA is digested on the column by adding DNase I.

Finally, a low-salt elution releases the RNA from the glass fiber. The process does not require RNA precipitation or organic solvent extraction, ideal for rapidly purifying many samples simultaneously.

Test Principle

1. Samples are disrupted in RNA Tissue Lysis Buffer and homogenized during an incubation with Proteinase K.
2. In the presence of chaotropic salt, nucleic acids (NA) bind specifically to the surface of glass fibers pre-packed in the High Pure Filter Tube.
3. To eliminate residual DNA, an on-column DNase I digestion is performed.
4. Bound RNA is washed, and thereby purified of salts, proteins, and other impurities.
5. For the final step, a low-salt elution releases the RNA from the glass fibers.

References

1 Lewis, N.J. Maughan, V. Smith, K. Hillan, P. Quirke, Unlocking the archive--gene expression in paraffin- embedded tissue, *J Pathol.* **195** (2001) 66-71.

2 N. Masuda, T. Ohnishi, S. Kawamoto, M. Monden, K. Okubo, Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples, *Nucleic Acids Res.* **27** (1999) 4436-4443

3 Vogelstein, B *et al.* (1979). Preparative and analytical purification of DNA from agarose. *Proc Natl Acad Sci USA* **76**, 615-619.

Quality control

Sections from a pre-characterized FFPET block are deparaffinized and isolated as described. RNA yield is determined by measuring the optical density at 260 nm and RNA purity is determined by the OD ratio 260/280 nm.

The integrity of the purified sample material is confirmed using the Agilent RNA Pico Kit. Absence of contaminating genomic DNA is examined by a PCR on a LightCycler® System without a reverse transcription step according to the current Quality Control Procedures.

6. Supplementary Information

6.1 Conventions

Text Conventions To make information consistent and easier to read, the following text conventions are used in this document:

Text Convention	Usage
Numbered stages labeled ①, ②, *etc.*	Stages in a process that usually occur in the order listed.
Numbered instructions labeled ❶, ❷, *etc.*	Steps in a procedure that must be performed in the order listed.
Asterisk *	Denotes a product available from Roche Applied Science.

Symbols In this document, the following symbols are used to highlight important information:

Symbol	Description
	Information Note: Additional information about the current topic or procedure.
⚠	Important Note: Information critical to the success of the procedure or use of the product.

6.2 Changes to Previous Version

Addition of new pack size.
Editorial Changes.

6.3 Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page, www.roche-applied-science.com, and our Special Interest Sites including: www.lightcycler.com, www.realtimeready.roche.com.

Product	Pack size	Cat. No.
High Pure FFPET DNA Isolation Kit	50 isolations	06 650 767 001
Transcriptor First Strand cDNA Synthesis Kit	1 Kit (50 reactions)	04 379 012 001
Transcriptor Universal cDNA Master	100 reactions	05 893 151 001
RealTime ready cDNA Pre-Amp Master	1 Kit (40 reactions)	06 720 455 001
RealTime ready Pre-Amp Primer Pool	1 Kit (6-96 primer pairs)	06 725 309 001
LightCycler® 480 Probes Master	5 × 1 ml (2× conc.)	04 707 494 001
RealTime ready Custom Panels	Visit: www.configurator.realtimeready.roche.com	
RealTime ready Custom Assays	Visit: www.configurator.realtimeready.roche.com	
RealTime ready Human Cell Cycle Regulation Panel	2 plates (each containing 96 assays)	05 339 359 001
RealTime ready Human Apoptosis Panel, 96	2 plates (each containing 96 assays)	05 392 063 001
RealTime ready Human Apoptosis Panel, 384	2 plates (each containing 384 assays)	05 339 316 001
RealTime ready Human Reference Gene Panel, 96	2 plates (each containing 96 assays)	05 339 545 001
RealTime ready Human Reference Gene Panel, 384	2 plates (each containing 384 assays)	05 467 675 001

6.4 Disclaimer of License

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Roche Diagnostics GmbH
Roche Applied Science
68298 Mannheim
Germany