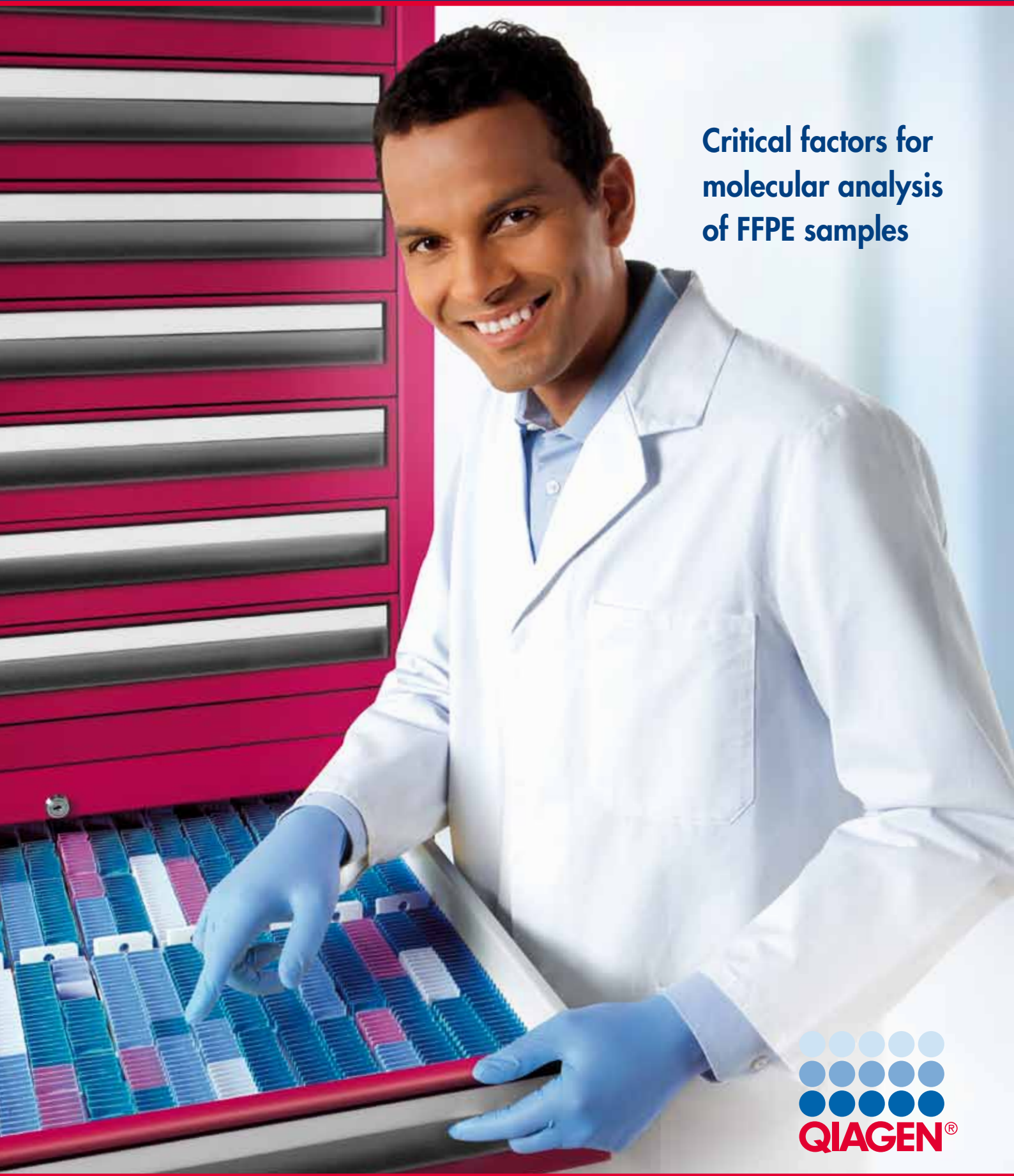
Unlocking your FFPE archive



Critical factors for molecular analysis of FFPE samples

QIAGEN®

Sample & Assay Technologies

Table of contents

Introduction

The archives of formalin-fixed, paraffin-embedded (FFPE) tissue sections stored around the world represent a valuable and extensive source of material for biomedical research. With more and more researchers turning towards molecular analysis of FFPE samples, it is becoming increasingly important to develop specific protocols that take into consideration the unique nature of these samples. This brochure provides advice on the complete FFPE workflow. Methods are described for preparation and storage of FFPE samples, with the aim of minimizing the reduction in quality of DNA, RNA, and protein caused by fixation and embedding processes. Next, the retrieval and analysis steps of the workflow are covered, with details of how to efficiently recover analyzable biomolecules from FFPE samples and what necessary modifications need to be made to downstream applications such as reverse transcription and real-time PCR.

Important considerations when preparing and archiving FFPE samples

The quality of the nucleic acids and proteins extracted from FFPE samples depends on how the samples were handled before, during, and after fixation and embedding. In this section, we identify the main factors that influence biomolecule quality and recommend what steps can be taken to minimize the effects of these factors.

2.1 Sample type

Each tissue has a specific physiological function and is unique with regard to its structure and constituent cells. Depending on the tissue type, the speed with which biomolecules are degraded, induced, or modified following harvesting can vary. Therefore, the procedures for tissue removal and fixation should be done as quickly as possible (see section 2.2). Special lysis conditions are not required when purifying analytes from fibrous or fatty FFPE tissues: instead, the presence of formalin crosslinks and paraffin determine the requirements for analyte retrieval (see section 3).

When working with tumorous tissues, it should be noted that healthy and malignant cells are not homogeneously distributed. Therefore, analysis of one section from a tissue sample may provide different results to analysis of another section from the same tissue sample.

2.2 Sample handling

The surgical procedure to obtain a tissue specimen from a patient involves administration of anesthetic, ligation of blood vessels, and excision of tissue, followed by fixation. During the time between patient anesthetization and tissue fixation, changes in gene expression in the tissue and tissue autolysis can occur. Therefore, the duration of the procedure prior to tissue fixation should be kept as short as possible to prevent significant changes in the profile of RNA transcripts and proteins. As a minimum, the duration of each step of the procedure should be recorded.

2.3 Formalin fixation

Fixation of tissues involves placing specimens in a formalin solution, which can vary in composition (a typical 10% formalin solution may contain 3.7% formaldehyde as well as 1–1.5% methanol). The resulting chemical reaction leads to crosslinks between biomolecules, including crosslinks between nucleic acids, between proteins, and between nucleic acids and proteins. For optimal results, neutral-buffered formalin solution should be used instead of unbuffered or acidic formalin solutions. Neutral buffer slows down the degradation of formalin, whose degradation products are believed to contribute to impairing nucleic acid quality.

Also important are 3 factors which influence the extent of tissue fixation: the thickness of the tissue specimen, the volume of the formalin solution, and the duration of the fixation process. Underfixation can lead to nucleic acid and protein degradation or a change in gene expression in deeper regions of the tissue specimen that have not been penetrated by formalin. Overfixation can result in more extensive crosslinking, which makes extraction of usable nucleic acids and proteins more difficult.

Formalin penetrates tissues at a rate of about 1 mm per hour. In addition, the rate of formalin penetration decreases with increase in tissue thickness. Therefore, when fixing a tissue specimen, it should be sufficiently thin to avoid overfixation at the periphery and underfixation at the center. In an experiment to compare fixation of differently sized samples, researchers at QIAGEN discovered significant RNA degradation when whole tissues (about 1 cm thick) were fixed instead of sliced tissues (thickness of 4 mm or less) (Figure 1). For optimal results, it is generally recommended to fix tissue specimens that are up to 5 mm thick.

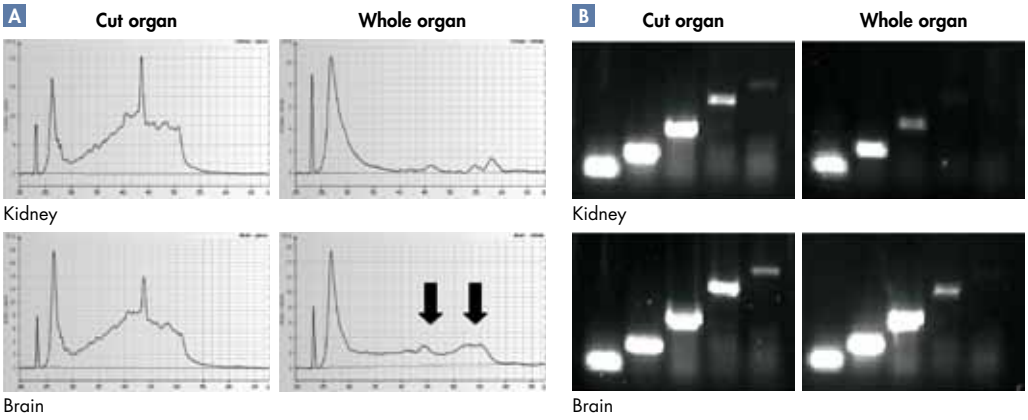


Figure 1. Effects of sample thickness on RNA integrity. Rat tissues were formalin-fixed, either as whole organs or as pieces ≤4 mm thick, and paraffin-embedded. A RNA was purified 3 days after embedding using the RNeasy® FFPE Kit and analyzed using the Agilent® 2100 Bioanalyzer. On the electropherogram for whole brain, the double arrows point to the reduced peaks for 18S rRNA and 28S rRNA, indicating increased RNA fragmentation. B The purified RNA was used in one-step RT-PCR with different primer pairs specific for the rat Rpl4 gene and the QIAGEN OneStep RT-PCR Kit. From left to right, the amplicon sizes are 96, 206, 400, 613, and 785 nucleotides. The absence of the larger amplicons with the whole organs indicates that greater RNA degradation had occurred. (Data excerpted from von Ahlfen et al. [2007] Determinants of RNA quality from FFPE samples. PloS ONE **12**, e1261.)

The ratio of formalin to tissue should be at least 10:1 to ensure optimal fixation. This is easy to achieve when working with small tissue specimens, such as needle biopsies. However, when dealing with large tissue samples there may be insufficient formalin for fixation. In this case, sections of the tissue should be cut for formalin fixation.

Tissues should be fixed for no more than 24 hours to avoid overfixation. In an experiment to compare overnight fixation with 72-hour fixation, QIAGEN scientists observed that fixation time had little effect on RNA integrity (Figure 2). However, they also found that 72-hour fixation had a negative impact on the performance of the purified RNA in one-step RT-PCR: larger amplicons could not be successfully amplified, which was most likely due to extensive crosslinking of RNA molecules and a higher proportion of irreversible crosslinks (Figure 1). Another experiment also revealed that a 72-hour fixation reduced the recovery of protein from FFPE samples (Figure 3). Again, this negative effect is probably due to increased crosslinking.

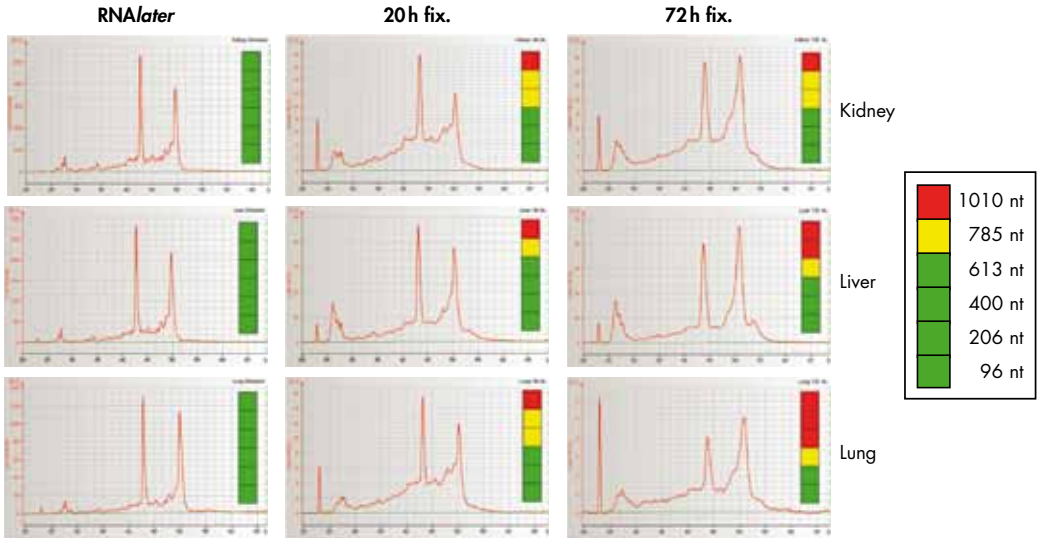


Figure 2. Effects of fixation time on RNA integrity and one-step RT-PCR. Various rat tissues were formalin-fixed, either overnight or for 3 days, and paraffin-embedded. For comparison, rat tissues were stabilized in RNA*later*® RNA Stabilization Reagent. RNA was purified 3 days after embedding using the RNeasy FFPE Kit and analyzed using the Agilent 2100 Bioanalyzer. In addition, one-step RT-PCR was carried out using the QIAGEN OneStep RT-PCR Kit and different primer pairs specific for the rat Rpl4 gene. The amplicon sizes are indicated in the figure, and the results of the RT-PCR are indicated by a color: red means no amplification; yellow means weak amplification; and green means successful amplification. Although the longer fixation time had only marginal effects on RNA fragmentation and ribosomal bands, it resulted in poorer efficiency of amplification of longer amplicons. (Data excerpted from von Ahlfen et al. [2007] Determinants of RNA quality from FFPE samples. PloS ONE **12**, e1261.)

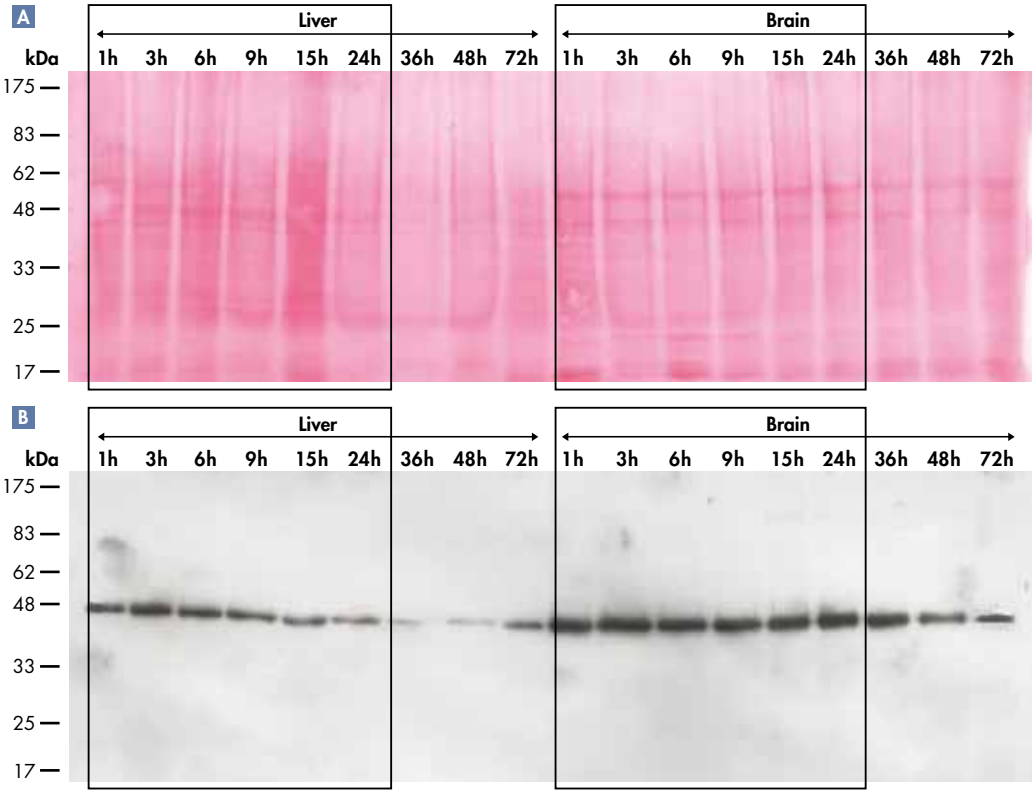


Figure 3. Effects of fixation time on protein extraction. Rat tissues were fixed with 10% neutral-buffered formalin for the indicated times and then embedded in paraffin. Total protein was extracted using the Qproteome® FFPE Tissue Kit. A SDS-PAGE analysis and B Western blot analysis using an antibody against β-actin were carried out. Both analyses show that a fixation time longer than 24 hours reduced the amount of protein extracted.

2.4 Paraffin embedding

After fixation in formalin, tissue specimens are embedded in paraffin, a process which consists of several steps. The first step is dehydration, where water is replaced by an alcohol, usually ethanol. This is followed by clearing, where the alcohol is replaced by xylene or a xylene substitute, and by impregnation, where xylene is replaced by paraffin. The final step is embedding, where the entire specimen is surrounded with paraffin. It is important that tissue specimens are fully dehydrated prior to impregnation, as residual water may lead to sample degradation. Paraffin embedding is a critical step for the maintenance of protein integrity, as residual water can lead to proteolysis. Therefore, high-quality reagents undiluted with water should be used, and the duration and temperature of the entire embedding process should be optimized to allow complete dehydration. We recommend always using fresh alcohol and xylene, to avoid any possibility of carryover of water from previous uses.

The paraffins used in impregnating and embedding formalin-fixed tissues vary in melting temperature and composition. When using high-melting temperature paraffin, higher temperatures are required for the embedding process, which can lead to increased sample degradation. To ensure optimal recovery of usable nucleic acids and protein from FFPE samples, low-melting-temperature paraffin should be used instead. In addition, paraffin containing additives such as beeswax should be avoided, as they may interfere with recovery of biomolecules.

2.5 Staining

To avoid problems in molecular analysis of FFPE samples, sample staining should be avoided if possible. Certain stains and reagents may adversely affect nucleic acids, especially those used for immunohistochemical staining. Staining procedures involving high pH and heavy metal ions should also be avoided. If sample staining is required, the use of cresyl violet stain is preferred, as it is compatible with nucleic acid analyses.

Purification of proteins from sections that have been stained using histology stains (e.g., hematoxylin or fast red) can result in dramatically decreased protein yield. However purified protein can be used for downstream analysis such as western blotting or reverse-phase protein microarray analysis.

2.6 Sample storage

After fixation and embedding, FFPE samples should ideally be stored at an optimal temperature that slows down the degradation of nucleic acids and proteins. In an experiment to compare different storage temperatures, scientists at QIAGEN found that RNA remains largely intact after 1 year if FFPE samples are stored at 4°C rather than at room temperature or higher (Figure 4).

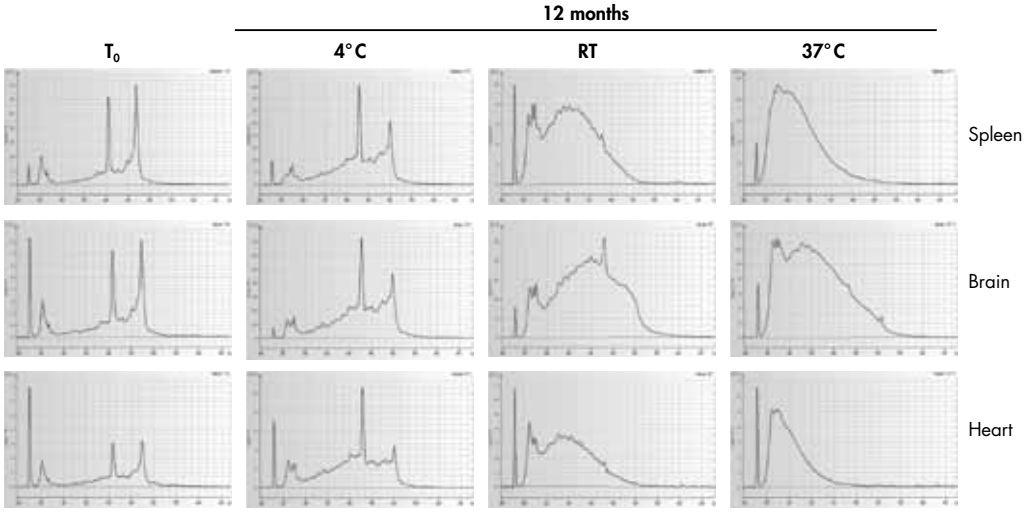


Figure 4. Effects of storage temperature on RNA integrity. Various rat tissues were formalin-fixed and paraffin-embedded. RNA was then purified using the RNeasy FFPE Kit, either 3 days after embedding (T_0) or after storage for 1 year at 4°C, 20–25°C, or 37°C. RNA integrity was analyzed on the Agilent 2100 Bioanalyzer. Peaks corresponding to 18S rRNA and 28S rRNA are clearly visible in the samples stored at 4°C, indicating intact RNA, but not in the samples stored at the higher temperatures. (Data excerpted from von Ahlfen et al. [2007] Determinants of RNA quality from FFPE samples. PloS ONE **12**, e1261.)

Since RNA in FFPE samples is often heavily fragmented, the chances of recovering largely intact RNA are greater for small RNAs such as microRNA (miRNA) than for longer RNAs such as mRNA. This means that assays to detect short amplicons (e.g., assays for specific miRNAs) are less compromised by formalin fixation than assays to detect long amplicons (e.g., assays for specific mRNA transcripts) (Figure 5).

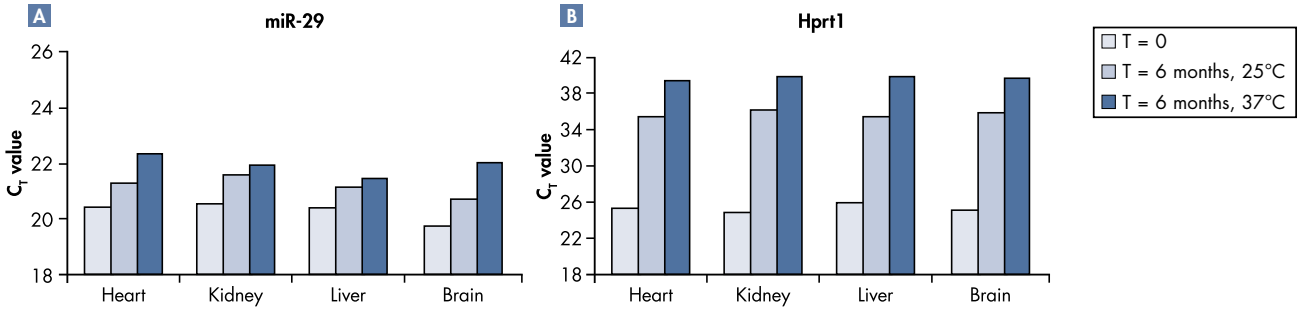


Figure 5. Effects of storage conditions on performance in real-time RT-PCR depend on RNA size. Various rat tissues were formalin-fixed and paraffin-embedded. Using the miRNeasy FFPE Kit, total RNA containing small and large RNAs was purified. The RNA samples were used as template in real-time RT-PCR using the miScript PCR System 3 days after embedding (T=0), 6 months after storage at room temperature, and 6 months after storage at 37°C. Reactions were performed on the ABI 7900 cycler. A The microRNA miR-29 (template length: 22 nt) and B the Hprt1 transcript (amplicon length: 94 bp) were detected. The 6-month storage had a greater effect on the performance of the mRNA assay than on that of the miRNA assay, indicating that RNA fragmentation had a greater effect on detection of longer templates than on detection of very short templates.

Critical factors for retrieval of usable analytes from FFPE samples

There are several major difficulties when purifying DNA, RNA, and protein from FFPE samples. Firstly, these biomolecules are crosslinked to each other. The extent of crosslinking and the proportion of irreversible crosslinks is dependent, at least in part, on the duration of formalin fixation (Figures 2 and 6). Secondly, nucleic acids are often heavily fragmented, depending on how the FFPE samples were prepared and stored. Thirdly, since FFPE samples are precious, there is only a limited amount of material which is available for analyte purification and downstream analysis. Therefore, the purification procedure used needs to be highly efficient, enabling the recovery of as much usable analyte as possible. This section describes the particular challenges in purifying DNA, RNA, and protein from FFPE samples and how best to overcome them.

3.1 Deparaffinization

Prior to purification of nucleic acids and proteins from FFPE samples, deparaffinization needs to be carried out. This is a process where paraffin is first dissolved and then removed, exposing samples for subsequent treatment with lysis buffer and, if appropriate, proteinase K. Various solvents are suitable for deparaffinization, with the choice of solvent depending on the purification procedure to be performed.

For purification of DNA or RNA involving digestion of samples with proteinase K and binding of nucleic acids to a silica membrane, various hydrophobic solvents can be used for deparaffinization. Examples include xylene, heptane, and limonene. For protein purification from FFPE samples, xylene deparaffinization is suitable if protein is to be used in downstream applications such as western blotting. However, for demanding applications such as 2D-PAGE, only heptane should be used to ensure highly efficient deparaffinization.

A more convenient method of deparaffinization prior to DNA or RNA purification from FFPE samples can be achieved using novel Deparaffinization Solution from QIAGEN. After addition to an FFPE sample, the solution remains on the sample while proteinase K digestion is carried out. There is no need to pellet the FFPE sample after addition of Deparaffinization Solution or to remove paraffin-containing supernatant. Thus, the risk of sample loss during deparaffinization is avoided.

Deparaffinization Solution

As an alternative to using a solvent, paraffin can be separated from an FFPE sample by heating: after melting, paraffin cools and, depending on the amount, sticks to the tube walls or forms a solid layer above the sample.

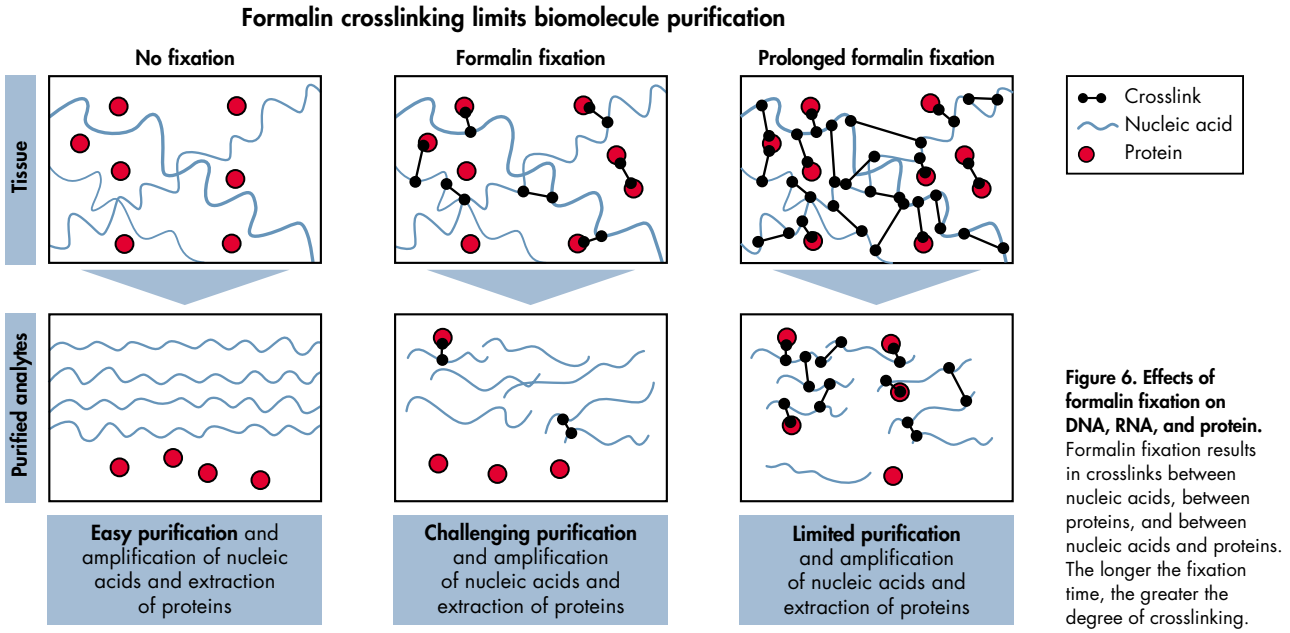


Figure 6. Effects of formalin fixation on DNA, RNA, and protein. Formalin fixation results in crosslinks between nucleic acids, between proteins, and between nucleic acids and proteins. The longer the fixation time, the greater the degree of crosslinking.

3.2 Purification of genomic DNA

Since FFPE samples contain DNA molecules that are crosslinked to each other as well as to RNA and protein molecules, breakage of these crosslinks is necessary in order to release DNA for subsequent purification. Chemical modifications due to crosslinking should also be reversed if possible, as chemically modified DNA is less efficiently recovered during the purification procedure and represents a poor substrate for PCR and other enzymatic assays. However, care should be taken when breaking crosslinks and reversing chemical modifications, as harsh reaction conditions may also lead to further DNA fragmentation.

QIAamp DNA FFPE Tissue Kit

An effective method for overcoming crosslinking is provided by the QIAamp® DNA FFPE Tissue Kit, which is specially designed for purification of genomic DNA from FFPE samples. Samples are initially treated with proteinase K at 56°C for 1 hour, which helps to release DNA by digesting crosslinked proteins. This is then followed by a 90°C incubation in an optimized buffer that partially reverses crosslinking: during this incubation, methylene bridges between amino groups are broken, and released formaldehyde molecules bind to acceptor molecules.

Although excessive heating can lead to DNA degradation, studies at QIAGEN reveal that the 90°C incubation for 1 hour enables optimal recovery of analyzable genomic DNA (Figures 7 and 8). The data also show that the 1-hour 56°C incubation followed by the 1-hour 90°C incubation negates the need for an overnight proteinase K digestion.

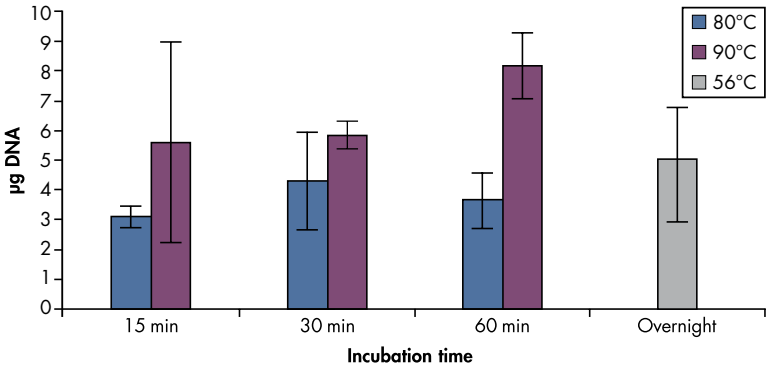


Figure 7. Optimal recovery of genomic DNA. FFPE sections of rat liver were treated with proteinase K at 56°C for 1 hour and then incubated as indicated. As a control, one sample was incubated overnight with proteinase K at 56°C. Genomic DNA was purified using the QIAamp DNA FFPE Tissue Kit, and DNA yields were determined using a spectrophotometer. The 90°C incubations enabled greater recovery of DNA than the 80°C incubations and the overnight incubation at 56°C.

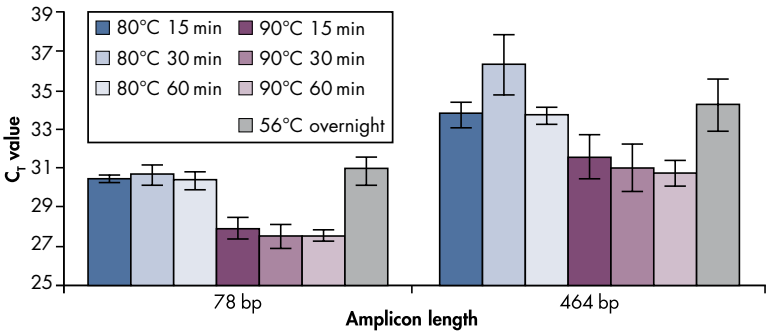


Figure 8. Recovery of usable genomic DNA from FFPE tissue. FFPE sections of rat liver were treated with proteinase K at 56°C for 1 hour and then incubated as indicated. Genomic DNA was purified using the QIAamp DNA FFPE Tissue Kit, and SYBR® Green-based real-time PCR was performed using the QuantiTect® SYBR Green PCR Kit and 2 different primer pairs specific for the Pmp2 gene. The resulting amplicons were either 78 bp or 464 bp in size. The 90°C incubations resulted in lower C_T values than the 80°C incubations: this indicates that the higher 90°C incubations were more effective in reversing crosslinking, providing more usable DNA for real-time PCR analysis. As a control, one sample was incubated overnight with proteinase K at 56°C.

After the 2 incubation steps, DNA is purified using QIAamp MinElute® spin columns: DNA is isolated on a silica membrane, washed to remove contaminants, and then eluted in a small volume of 20–100 µl. Optionally, RNase A digestion of the sample can be performed prior to binding of DNA to the silica membrane. This step may be necessary if the DNA will be analyzed in downstream applications that are sensitive to RNA contamination. RNase A digestion is also necessary if the purified DNA needs to be quantified spectrophotometrically, since RNA contamination contributes to absorbance readings and leads to overestimation of DNA yields. For increased standardization and convenience, DNA purification using the QIAamp DNA FFPE Tissue Kit can be automated on the QIAcube® if desired (see section 3.8).

Although optimal lysis and incubation conditions can release DNA from FFPE samples and partially reverse formaldehyde modification, there are some limitations with using the purified DNA in downstream assays. Firstly, chemical modification during the fixation process may introduce unwanted sequence changes, reduce DNA stability and the efficiency of enzymatic assays, and make analysis of purity by A_{260}/A_{280} measurements difficult. Secondly, since DNA in FFPE samples becomes increasingly fragmented over time, it is only possible to analyze short sequences (e.g., in PCR applications, primers to produce short amplicons should be designed). In addition, shorter DNA fragments may lead to overestimation of DNA yield due to higher absorbance readings.

Note: The QIAamp DNA FFPE Tissue Kit is intended for purification of DNA from samples fixed using formalin. It does not work with samples that have been fixed with other reagents that completely destroy DNA, such as picric acid.

3.3 Whole genome amplification

The precious nature of FFPE samples means that only small amounts of DNA can be purified, which limits the number of molecular analyses that can be carried out. Increasing the amount of a DNA sample can be achieved by Whole Genome Amplification (WGA), where the entire genome is amplified using a highly processive DNA polymerase. However, this is a challenge when starting with fragmented DNA from FFPE samples.

REPLI-g FFPE Kit

The REPLI-g® FFPE Kit uses an adapted WGA method to allow amplification of genomic DNA from FFPE samples, and requires no prior DNA purification. Fragmented DNA is first randomly ligated to generate DNA molecules of a higher molecular weight. WGA is then carried out using a highly processive DNA polymerase. Although the DNA fragments have not been reassembled in the correct order, reliable DNA analysis is possible with certain applications, provided modifications to the method are made. For example, PCR and real-time PCR analyses can be performed if primers are designed to generate amplicons that are as short as possible (Figure 9). Although the data show successful PCR amplification of a 358 bp fragment, we recommend designing PCR assays to generate amplicons of around 100 bp, since DNA in FFPE samples can be heavily fragmented and since formaldehyde modification reduces the length of readable DNA sequence.

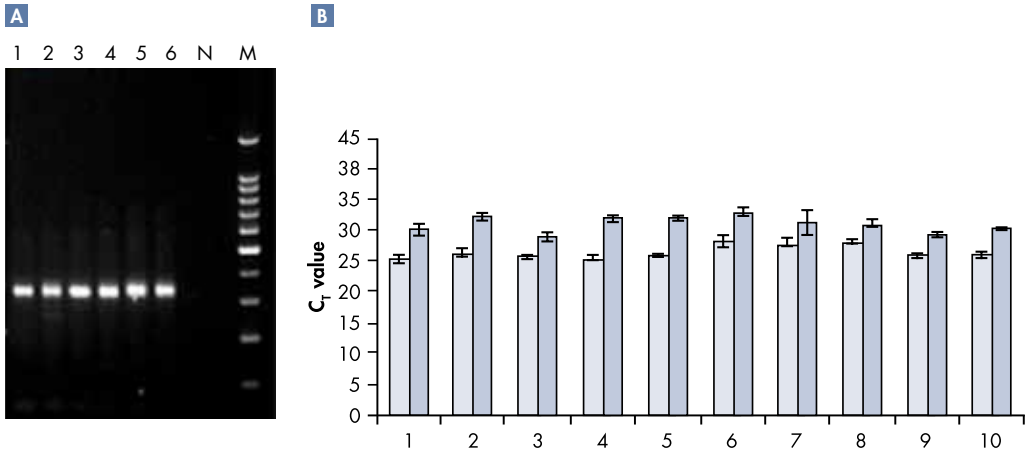


Figure 9. Reliable PCR and real-time PCR using genomic DNA from FFPE tissue. A Six samples of FFPE human liver (11 years old) were subjected to WGA using the REPLI-g FFPE Kit. The resulting DNA samples were then used in end-point PCR using the QIAGEN Fast Cycling PCR Kit on an Eppendorf® Mastercycler® ep gradient S to amplify a 358 bp sequence. **1**–**6**: DNA samples; **N**: no template control; **M**: markers. B Two samples of FFPE human liver (11 years old) were subjected to 10 independent WGA reactions using the REPLI-g FFPE Kit. Real-time PCR using the QuantiFast SYBR Green PCR Kit and a Stratagene® Mx3005P® cycler was then performed to amplify a 100 bp sequence.

3.4 Bisulfite conversion

EpiTect Plus FFPE Bisulfite Kit

The first step in epigenetic DNA methylation analysis is to carry out bisulfite conversion, where unmethylated cytosines are converted to uracils. Since methylated cytosines remain unaffected by bisulfite conversion, they can be detected by PCR or real-time PCR, or detected and quantified by Pyrosequencing®. However, since bisulfite conversion requires incubation at high temperatures and at low pH values, fragmentation of DNA can occur. This can be a problem when working with DNA from FFPE samples that is already heavily fragmented.

The EpiTect® Plus FFPE Bisulfite Kit combines a number of unique features to enable optimal recovery of DNA from FFPE samples and to provide maximum bisulfite conversion without further DNA degradation. Firstly, the kit uses an optimized deparaffinization step, which minimizes handling, saves time, and maximizes DNA yield. After deparaffinization, samples are initially treated with proteinase K at 56°C for 30 minutes to release DNA by digesting crosslinked proteins. This is then followed by a 95°C incubation for 1 hour in a novel buffer to partially reverse crosslinking: during this incubation, methylene bridges between amino groups are broken. Although excessive heating can lead to DNA degradation, studies at QIAGEN reveal that the 95°C incubation for 1 hour enables optimal recovery of genomic DNA, which can then be used in the bisulfite conversion step without further purification. Chemical DNA degradation during bisulfite conversion is prevented by innovative DNA Protect technology.

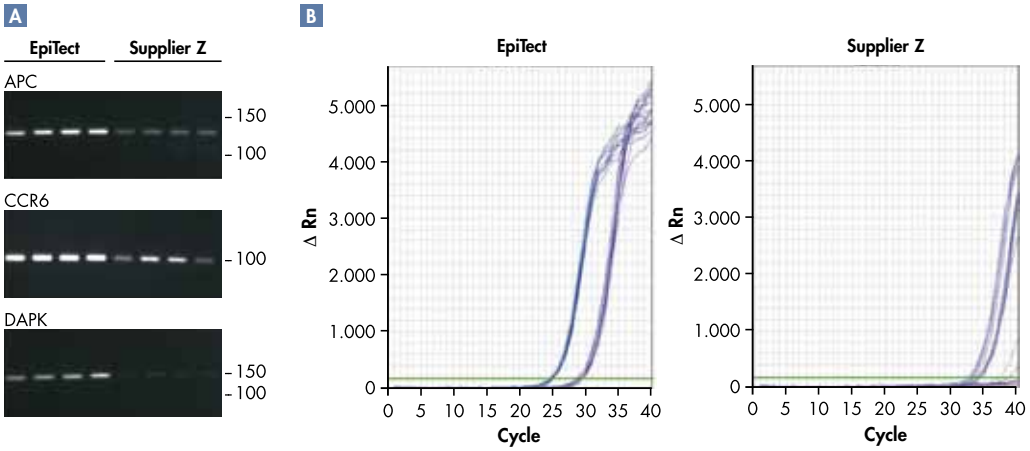


Figure 10. PCR analysis after bisulfite conversion of DNA from FFPE samples. DNA was purified from rat liver FFPE tissue (10 µm) using the EpiTect Plus FFPE Bisulfite Kit or an alternative kit from Supplier Z. A End-point PCR was performed to detect the indicated genes using the PyroMark® PCR Kit. Agarose gel analysis showed superior and highly consistent yields from templates purified using the EpiTect Plus FFPE Bisulfite Kit compared to templates purified with the alternative kit. B Quantitative real-time PCR was performed using the QuantiTect SYBR Green PCR Kit to detect 2 amplicons of different size (156 and 279 bp) of the GSTP gene. Each reaction was performed for 4 samples, in triplicate. The left amplification plot shows lower C_T values and highly reproducible results for both amplicons from templates purified using the EpiTect Plus FFPE Bisulfite Kit. Only the 156 bp fragment was successfully amplified from templates purified using the alternative kit, and this with higher C_T values (right).

3.5 Purification of total RNA and miRNA

RNeasy FFPE Kit

miRNeasy FFPE Kit

The problem of formaldehyde modification which affects DNA in FFPE samples (see section 3.2) also applies to RNA as well. The new, optimized RNeasy FFPE Kit and miRNeasy FFPE Kit are both specially designed to purify RNA from FFPE samples (Figures 11 and 12). Since RNA is more easily fragmented by heat treatment, optimized buffer conditions are used and incubation temperatures are kept lower. While incubation above 80°C may slightly improve RNA performance in RT-PCR, it will also result in greater RNA fragmentation. Also, incubation at lower temperatures will result in lower yields and significantly poorer performance in RT-PCR.

FFPE samples are first digested with proteinase K at 56°C for 15 minutes to release RNA molecules from crosslinked protein molecules, and then incubated at 80°C for 15 minutes to reverse some of the formaldehyde modification. Next, to avoid DNA contamination, and particularly to remove even trace amounts of small DNA fragments which can impair downstream assays such as real-time RT-PCR, RNA is treated with DNase Booster and DNase. In the following steps, RNA is purified using an RNeasy MinElute spin column, where RNA is bound, washed, and finally eluted in 15–30 µl of RNase-free water. Depending on the binding conditions, either total RNA down to about 70 nucleotides (RNeasy FFPE Kit) or total RNA including miRNA down to about 18 nucleotides (miRNeasy FFPE Kit) is purified. Due to the fragmented nature of the RNA, it is not practical to attempt to purify a separate, small-RNA–enriched fraction. For increased standardization and convenience, RNA purification using the RNeasy and miRNeasy FFPE Kits can be automated on the QIAcube if desired (see section 3.8).

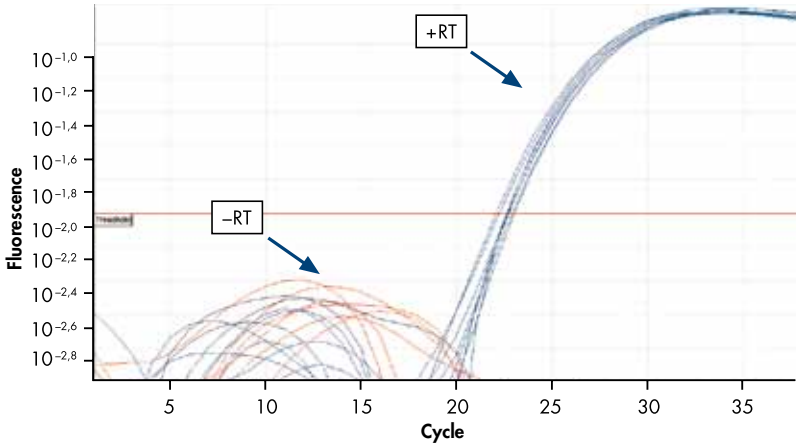


Figure 11. Real-time RT-PCR analysis of RNA from FFPE samples. Total RNA was purified from rat kidney using the RNeasy FFPE Kit. Real-time RT-PCR assays for PGK1 (phosphoglycerate kinase 1) were performed on the Rotor-Gene® Q cycler with (**+RT**) or without (**–RT**) reverse transcriptase. The –RT curves demonstrated that RNA purified using the RNeasy FFPE Kit was virtually free of genomic DNA.

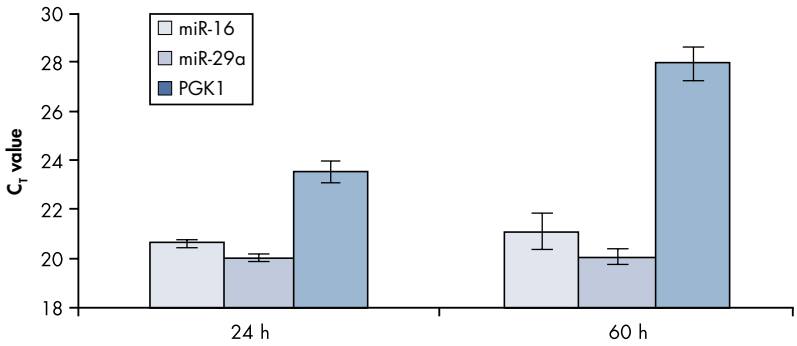


Figure 12. Efficient miRNA purification from FFPE tissues. Rat liver tissue was formalin fixed for 24 hours or 60 hours, followed by purification of total RNA including miRNA using the miRNeasy FFPE Kit. Purified RNA was used as a template in quantitative, real-time RT-PCR using the miScript PCR System to detect miRNAs miR-16 and miR-29a and the larger mRNA of the PGK1 gene. Results show successful detection of both miRNAs as well as the mRNA from the same eluate.

3.6 Purification of protein

Unlike nucleic acids, proteins in FFPE samples do not suffer the problem of being fragmented. Therefore, the isolation of full-length proteins is possible. However, there are several important issues when extracting proteins from FFPE samples. Firstly, thorough removal of paraffin is required if sample lysate rather than highly purified protein is required for downstream applications. Secondly, proteinase K and highly denaturing agents cannot be used to break formalin crosslinks, as they will lead to protein degradation.

The efficiency of protein purification is influenced by the thickness of the sections. We recommend a sample thickness of 10–15 µm. The yield depends on the amount of tissue in the sections and the nature of tissue. Typical yields obtained from three 10 µm sections are 1.5 µg/µl from liver tissue and 1.6 µg/µl from brain tissue.

Qproteome FFPE Tissue Kit

The Qproteome FFPE Tissue Kit uses proprietary technology to efficiently recover full-length proteins from FFPE samples. After multiple treatments with xylene to remove paraffin, tissue sections undergo 2 incubations in an optimized extraction buffer. The first is at 100°C for 20 minutes to reverse formalin crosslinking. The second is at 80°C for 2 hours to ensure maximal protein solubilization. A protein-containing lysate with a volume of 100 µl is obtained, suitable for applications such as mass spectrometry and western blot analysis (Figure 13).

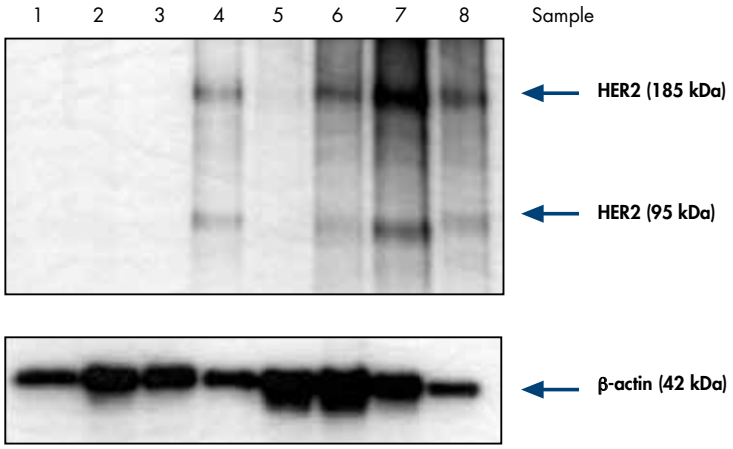


Figure 13. Detection of HER2 in breast cancer biopsies. Eight FFPE breast tissue samples were processed using the Qproteome FFPE Tissue Kit and proteins separated by SDS-PAGE. After western blotting, blots were probed with HER2 (upper panel) or β-actin (lower panel, control) antibodies. Both p185 and p95 forms of HER could be detected in the protein samples extracted from FFPE tissues. (Data kindly provided by Karl-Friedrich Becker and Christina Schott, Technical University of Munich, Germany.)

Extraction of proteins suitable for 2D-PAGE is provided by the Qproteome FFPE Tissue 2D-PAGE Kit. After a deparaffinization step specifically developed for 2D-PAGE samples, formalin crosslinking is reversed using the same extraction buffer as that supplied with the Qproteome FFPE Tissue Kit. The recovered proteins are then desalted to remove salts that could affect downstream 2D-PAGE analysis. Purified proteins are also suitable for use in mass spectrometry analysis (Figure 14).

Qproteome FFPE Tissue 2D-PAGE Kit

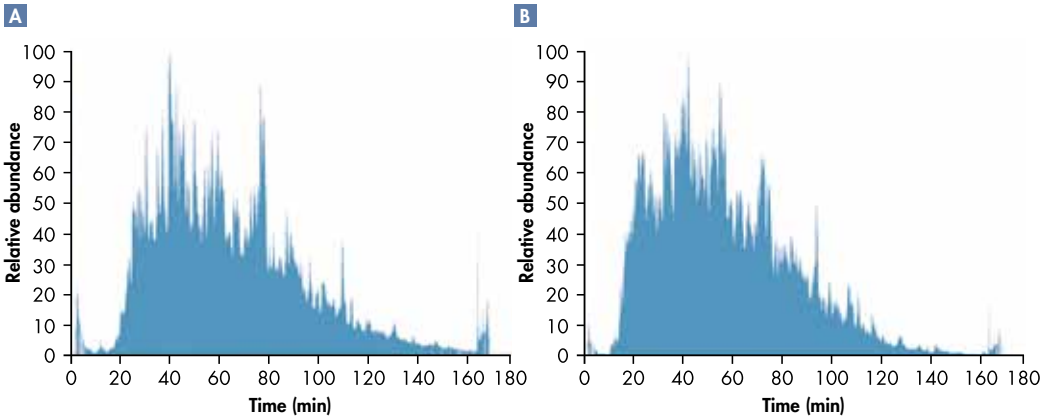


Figure 14. Mass spectrometry analysis of proteins from FFPE and fresh-frozen samples. Protein was purified from FFPE and fresh-frozen rat liver using the Qproteome FFPE Tissue 2D-PAGE Kit. Protein yield from the FFPE tissue was 80 µg from three 150 mm^2 sections. Total ion chromatograms of LC-coupled tandem MS analysis of A hydrolyzed FFPE and B fresh liver tissue are shown. The LC gradient applied was 5% to 40% solvent B over 160 min. The analysis was performed on an Orbitrap™ XL mass spectrometer (Thermo Fisher Scientific) by applying 5 µg of tryptic peptide mixture derived by enzymatic digestion of proteins extracted from A FFPE or B fresh-frozen tissue to an HPLC column coupled to the mass spectrometer. Approximately 86.7% of the total proteins were identified in both the freshly frozen and the FFPE tissue when the same amount of starting material was analyzed. (Data excerpted from Geoui, T. et al. [2010] Extraction of Proteins from Formalin-Fixed, Paraffin-Embedded Tissue Using the Qproteome Extraction Technique and Preparation of Tryptic Peptides for Liquid Chromatography/Mass Spectrometry Analysis. Curr. Protoc. Mol. Biol. **90**, 10.27.1).

3.7 Simultaneous purification of multiple analytes

Reliable comparison of genomic and transcriptomic data is achieved if both DNA and RNA are purified from the same FFPE sample. This is particularly important when working with tumorous tissues, which contain a heterogeneous distribution of healthy and malignant cells: this means that different sections from the same sample may differ in their cellular composition. Simply dividing a sample in half for separate DNA and RNA purification procedures results in the purification of DNA and RNA from different populations of cells, which may differ in their properties. Purification of DNA and RNA from the same sample also helps to prevent wastage, since FFPE samples are precious, often difficult to retrieve, and limited in amount.

AllPrep DNA/RNA FFPE Kit

However, there is a major obstacle to isolating DNA and RNA from the same sample: fragmented DNA is short and can be partly single-stranded and therefore more closely resembles RNA than intact DNA. This property of fragmented DNA makes physical separation of DNA and RNA difficult. The AllPrep® DNA/RNA FFPE Kit uses a patent-pending solubilization method to differentially release DNA and RNA from a single FFPE sample. With this method, FFPE samples are incubated in an optimized lysis buffer, which results in the release of RNA and precipitation of DNA. After centrifugation, the RNA-containing supernatant and DNA-containing pellet are then processed separately to purify RNA and DNA. Further incubations partially reverse crosslinking, and RNA or DNA is then purified using an RNeasy MinElute spin column or QIAamp MinElute spin column. For purified RNA, an on-column DNase treatment efficiently removes any contaminating DNA (Figure 16C).

Depending on the RNA binding conditions, small RNAs such as miRNA are either absent or present in the purified RNA. For purified DNA, an on-column RNase treatment is optional, as RNA contamination is minimal due to the separation of DNA and RNA prior to spin column processing.

DNA and RNA purified using the AllPrep DNA/RNA FFPE Kit are of comparable quality to DNA and RNA purified using the QIAamp DNA FFPE Tissue Kit and RNeasy FFPE Kit/miRNeasy FFPE Kit, respectively (Figure 15). The purified nucleic acids are therefore suitable for downstream applications such as real-time PCR and RT-PCR (Figure 16) or Pyrosequencing.

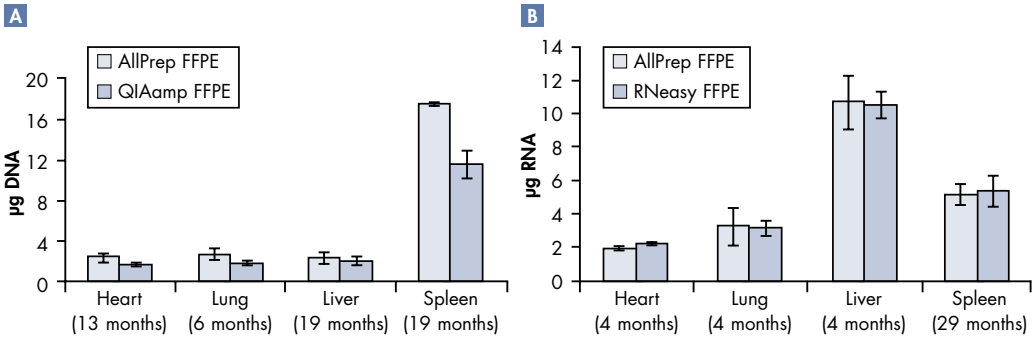


Figure 15. Purification of DNA and RNA from FFPE samples. A Genomic DNA was purified from various FFPE rat tissues that were stored at room temperature for the times indicated. Purification was performed using either the AllPrep DNA/RNA FFPE Kit or, as a control, the QIAamp DNA FFPE Tissue Kit (a dedicated kit for DNA purification from FFPE samples) both including RNase digestion. DNA yields from 20 µm sections of each sample were determined by absorbance measurement. B RNA was purified from various FFPE rat tissues that were stored at room temperature for the times indicated. Purification was performed using either the AllPrep DNA/RNA FFPE Kit or, as a control, the RNeasy FFPE Kit (a dedicated kit for RNA purification from FFPE samples). RNA yields from 10 µm sections of each sample were determined by absorbance measurement. The AllPrep Kit performed just as well as the dedicated DNA/RNA purification kits in recovering DNA/RNA from FFPE samples.

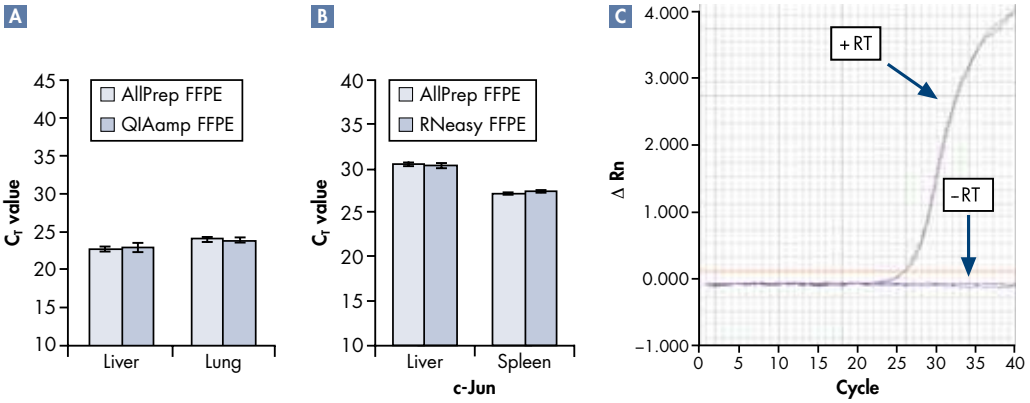


Figure 16. Reliable amplification of DNA and RNA from FFPE samples. A DNA and B, C RNA were purified from various FFPE rat tissues using either the AllPrep DNA/RNA FFPE Kit or, as a control, dedicated kits for DNA or RNA purification from FFPE samples (QIAamp DNA FFPE Tissue Kit or RNeasy FFPE Kit). Real-time PCR or RT-PCR was carried out on an ABI PRISM® 7900HT Sequence Detection System using A the QuantiTect SYBR Green PCR Kit to analyze a 78 bp amplicon of the Prnp gene or B, C the QuantiTect SYBR Green RT-PCR Kit to analyze Jun oncogene expression. The AllPrep Kit and the dedicated kits provided comparable C_T values, indicating that all kits achieved similar efficiency in recovering usable DNA or RNA. C In addition, analysis of Jun expression was carried out without reverse transcriptase (**–RT**). The amplification plot for the spleen sample, a DNA-rich tissue, indicated the virtual absence of genomic DNA contamination.

3.8 Automation of retrieval and purification

Automation of FFPE analyte purification offers the advantages of increased standardization of the purification process and reduced labor. It is also beneficial for processing multiple samples. The QIAcube automates QIAGEN spin column kits, including the QIAamp DNA FFPE Tissue Kit (section 3.2), the RNeasy FFPE Kit, and the miRNeasy FFPE Kit (section 3.5) enabling fast start-up of low-throughput automated purification (up to 12 samples per run). For low- to medium-throughput DNA purification from FFPE samples, EZ1® Advanced instruments purify from 1–6 or 1–14 FFPE samples per run using the EZ1 DNA Paraffin Section Card and the EZ1 DNA Tissue Kit. EZ1 Advanced instruments provide high process safety, effortless data management, and fast run times.

QIAcube

EZ1 Advanced

Critical factors for successful molecular analysis of FFPE samples

As described earlier in this brochure, formalin fixation and paraffin embedding can impair the quality of nucleic acids obtained from tissue samples. In this section, we explain how molecular analysis of FFPE samples is negatively affected by the low quality of analytes from FFPE samples and recommend the necessary steps to overcome this challenge.

4.1 RNA quality control

QIAxcel System

When performing quality control of an RNA sample, the parameters that are typically measured include the ratio of absorbances at 260 nm and 280 nm (pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1 in 10 mM Tris·Cl, pH 7.5) and the ratio of 28S rRNA to 18S rRNA (a ratio of 2:1 indicates intact RNA). Analysis of RNA samples by capillary electrophoresis, for example using the QIAxcel® System, indicates the fragmentation state of the RNA (Figure 17). The QIAxcel enables analysis of up to 96 RNA FFPE samples without manual intervention. It provides faster analysis times than alternative quality control systems, as well as easier handling due to ready-to-run gel cartridges.

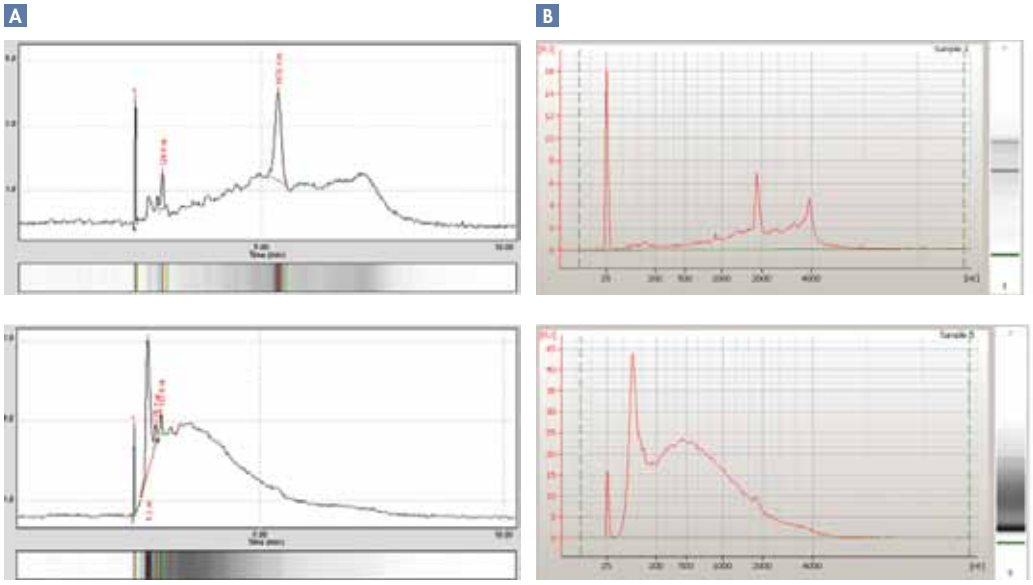


Figure 17. Quality control analysis of RNA from FFPE samples. Rat kidney samples were formalin-fixed and embedded in paraffin. Sections were cut directly after embedding (upper graphs), or after 12 months storage (lower graphs). Total RNA was purified using the RNeasy FFPE Kit. Purified RNA was analyzed A on the QIAxcel or B on the Agilent 2100 BioAnalyzer.

Systems such as the Agilent 2100 Bioanalyzer also yield a RIN (RNA Integrity Number) or equivalent value, which can range from 1 to 10. A RIN value of 10 indicates highly intact RNA, whereas a RIN value of 1 indicates highly fragmented RNA. While these parameters give an idea of RNA purity and integrity, they do not provide information about any chemical modifications. As discussed earlier, formalin fixation introduces crosslinks between molecules, and modification of RNA by formaldehyde can impair downstream enzymatic assays such as RT-PCR (Figure 18).

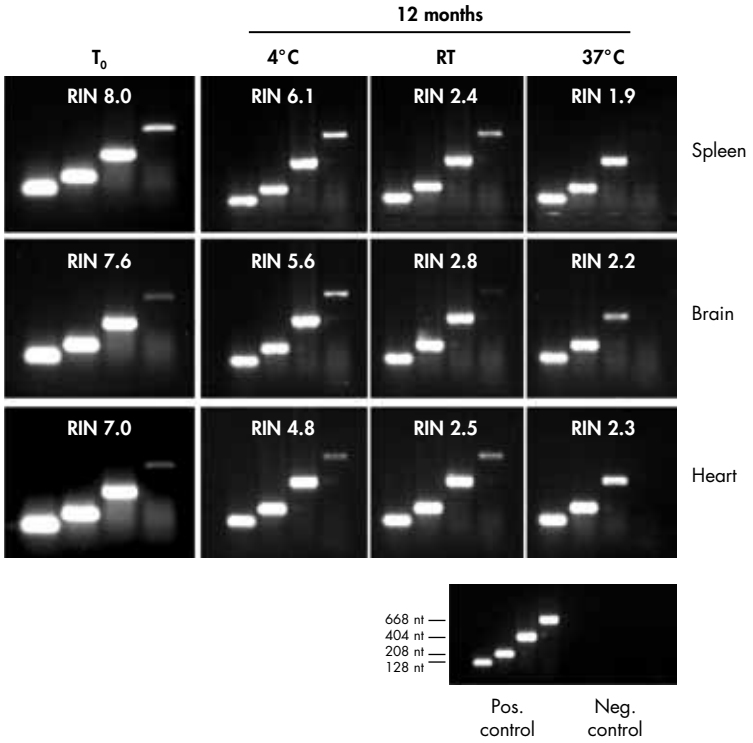


Figure 18. Effect of formalin fixation and paraffin embedding on RT-PCR efficiency. Various rat tissues were formalin fixed and paraffin embedded. RNA was then purified using the RNeasy FFPE Kit, either 3 days after embedding (T_0) or after storage for 1 year at 4°C, 20–25°C, or 37°C. RNA integrity was analyzed on the Agilent 2100 Bioanalyzer and the RINs are indicated above and electropherograms are shown in Figure 4, page 9. The purified RNA was used in one-step RT-PCR using the QIAGEN OneStep RT-PCR Kit and different primer pairs specific for the rat Hprt1 gene, yielding amplicons that were 128, 208, 404, and 668 nucleotides long. As a positive control, RT-PCR was carried out using RNA from fresh tissues, and as a negative control, RT-PCR was performed with no template. The results show that amplification of the longer amplicons was impaired in the FFPE samples. In addition, PCR performance did not correlate with RNA integrity. This is because for most samples, chemical modification of the RNA by formaldehyde is limiting, and not RNA fragment length. (Data excerpted from von Ahlfen et al. [2007] Determinants of RNA quality from FFPE samples. PloS ONE **12**, e1261.)

Therefore, assessment of the quality of RNA from FFPE samples should include a set of RT-PCR assays to determine the upper limit with regard to amplicon size. Alternatively, in the case of oligo-dT–primed cDNA, real-time RT-PCR assays can be carried out using different primer pairs to generate similar sized amplicons located at increasing distance from the 3' end of an RNA transcript. The degree of success of the amplification reactions would give an indication of the extent of RNA degradation along the entire transcript. Since oligo-dT priming is not recommended for RNA from FFPE samples (see section 4.3), the first approach is preferred.

With regard to analysis of highly fragmented RNA samples using the Agilent 2100 Bioanalyzer, RIN values of around 4 or below are not very informative or reproducible in our experience. For strongly degraded RNA, we believe it would be better to use peak fragment length (which can be deduced from the electropherogram) as a measure of RNA integrity.

4.2 DNA analysis by PCR and real-time PCR

As described earlier in this brochure, genomic DNA in FFPE samples is often heavily fragmented and also modified through the introduction of formalin crosslinks. Purification of genomic DNA with the QIAamp DNA FFPE Tissue Kit does reverse some of the crosslinks, but other crosslinks are irreversible and remain present after purification. Therefore, a robust PCR system is necessary for efficient amplification of this difficult template type. Since only a fraction of the starting template will be amplifiable, high PCR sensitivity and specificity are prerequisites for successful detection from FFPE samples. HotStarTaq® DNA Polymerase and HotStarTaq Plus DNA polymerase in combination with QIAGEN's unique PCR Buffer provide highly sensitive PCR from this type of starting template. In addition, it is highly recommended to design the amplicons to be as short as possible when carrying out PCR or real-time PCR analysis of genomic DNA from FFPE samples.

4.3 Gene expression analysis by real-time RT-PCR

Analysis of gene expression can be achieved by real-time RT-PCR, where RNA purified from a sample is first converted to cDNA by reverse transcription, and the desired cDNA target is then amplified and detected in real-time.

QuantiFast Probe RT-PCR Plus Kit

The presence of genomic DNA contamination in an RNA sample affects the accuracy of gene expression analysis by real-time RT-PCR if the primers used amplify both cDNA and genomic DNA sequences. Therefore, elimination of genomic DNA contamination is essential for accurate results. This can be achieved by purifying RNA using the RNeasy FFPE Kit, miRNeasy FFPE Kit, or AllPrep DNA/RNA FFPE Kit, which include a step to remove trace amounts of even small fragments of genomic DNA in isolated RNA. In addition, real-time RT-PCR can be performed as a two-step application using the QuantiTect Reverse Transcription Kit for cDNA synthesis, followed by amplification using a QuantiFast PCR Kit. The QuantiTect Reverse Transcription Kit uses novel gDNA Wipeout technology to remove genomic DNA contamination during cDNA synthesis. Alternatively, real-time RT-PCR can be performed as a one-step application with the QuantiFast® Probe RT-PCR Plus Kit, which removes genomic DNA contamination prior to the start of real-time RT-PCR. With the QuantiFast Probe RT-PCR Plus Kit, the C_T values obtained can be significantly higher than those achieved with other methods depending on the genomic DNA content and the level of gene expression. This is not because the kits provide lower PCR sensitivity, but because the kits allow detection of cDNA only instead of detection of cDNA as well as genomic DNA (Figure 19).

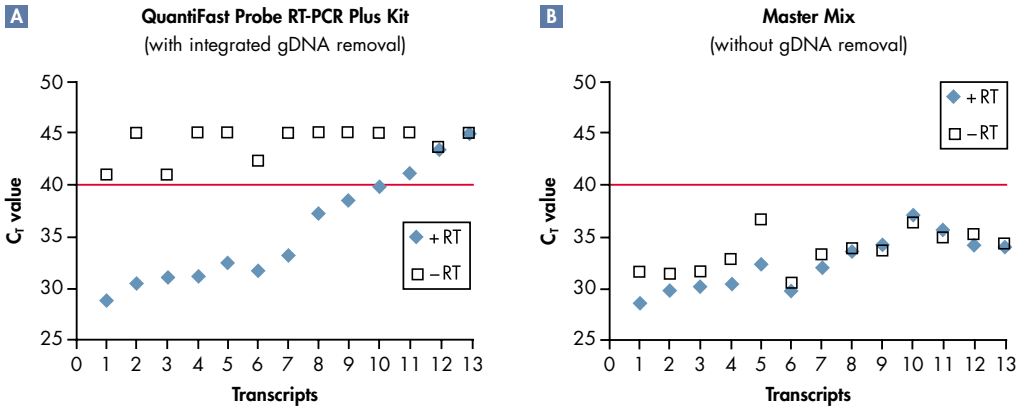


Figure 19. Efficient genomic DNA removal for accurate gene expression analysis. Total RNA was purified from human breast, liver, or kidney FFPE samples with the RNeasy FFPE Kit. Duplex, real-time RT-PCR was performed with **(+RT)** or without **(–RT)** the reverse transcription step for a range of different targets with different expression levels using QuantiFast Probe Assays (FAM™ labeled) and A the QuantiFast Probe RT-PCR Plus Kit or B an alternative kit for multiplex PCR which does not include an integrated gDNA removal step. All reactions were performed on the ABI StepOnePlus™ cycler. A High C_T values in the –RT samples indicate no amplification and the absence of genomic DNA contamination. Therefore the C_T values obtained in the +RT samples reflect reliable and accurate detection of RNA. Targets 12 (TUSC2) and 13 (EZH2) are not expressed. These results show that the QuantiFast Probe RT-PCR Plus Kit efficiently removes genomic DNA. B Similar C_T values in both the –RT and +RT samples indicate that contaminating genomic DNA is being detected, making these results unreliable.

In the reverse-transcription reaction, the most commonly used primers are either random hexamers or oligo-dT primers. However, these primers are not ideal for use with chemically modified and fragmented RNA from FFPE tissues. With oligo-dT primers, reverse transcription starts from the poly-A tail of mRNA transcripts. Therefore, if the RNA is heavily fragmented or modified by formaldehyde, only cDNA corresponding to the 3' end of transcripts will be synthesized. Random hexamers are more suitable, especially for profiling studies (e.g., microarray analysis). However they also have limitations, particularly if studying the expression of single genes. Only the random hexamers that are close to the amplicon of interest will generate the cDNA target required for real-time PCR analysis. As these hexamers make up only a small percentage of the overall number of hexamers in the reverse-transcription reaction, their priming efficiency will be very low.

QuantiFast Probe Assays

For optimal results in real-time RT-PCR, gene-specific primers close to the amplicon of interest should be used for the reverse-transcription step. Alternatively, a mixture of oligo-dT primers and random oligomers as contained in the QuantiTect Reverse Transcription Kit should be used. In addition, primers for the real-time PCR step should be designed to produce amplicons that are as short as possible. If amplicons chosen are longer than the RNA fragments they will not amplify, leading to inaccurate results. The size of RNA fragments from typical FFPE samples typically ranges from 50 to 300 nucleotides, with most fragments being around 100 nucleotides in size. QuantiFast Probe Assays, based on well-established hydrolysis probe technology (5' nuclease technology, e.g., TaqMan®), are designed using a proprietary algorithm for gene expression analysis of FFPE samples by probe-based real-time RT-PCR. QuantiFast Probe Assays enable amplification and detection of RNA and cDNA targets less than 100 bp in size with high efficiency and reliability (Figure 20).

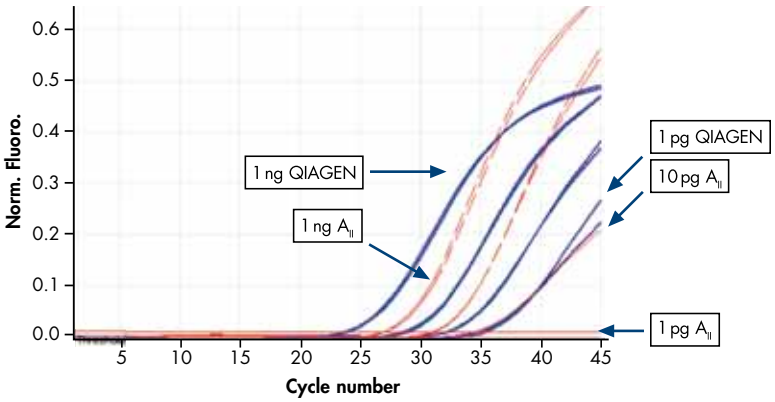


Figure 20. Highly efficient real-time RT-PCR detection using RNA from FFPE sample. Total RNA (1 ng, 100 pg, 10 pg, and 1 pg) was purified from a breast tissue FFPE sample using the RNeasy FFPE Kit. Transcripts of the human MUC1 gene were amplified and detected on the Rotor-Gene Q cycler using the QuantiFast Probe RT-PCR Plus Kit and the QuantiFast Probe Assay (blue curves) or a predesigned assay from Supplier A (red curves). Results from the QuantiFast Probe Assay showed lower C_T values and higher sensitivity compared to results using the assay from Supplier A. As low as 1 pg template was detectable using the QuantiFast Probe Assay (1 pg was undetectable using the assay from Supplier A).

RT² FFPE PreAMP technology

The precious nature of FFPE samples means that only a small amount of RNA can be recovered, limiting the number of analyses by real-time RT-PCR. This can be a major problem when a multitude of genes needs to be analyzed. To increase the amount of cDNA targets available for analysis, a preamplification step can be introduced between the reverse-transcription and real-time PCR steps. With RT² FFPE PreAMP technology, multiplex PCR is carried out following cDNA synthesis to preamplify cDNA targets derived from genes specific for a particular pathway or disease state. After preamplification, each cDNA target is individually quantified by real-time PCR using the appropriate RT² Profiler PCR Array (Figure 21). RT² FFPE PreAMP technology not only increases the sensitivity of real-time PCR analysis, but also preserves the original gene expression profile.

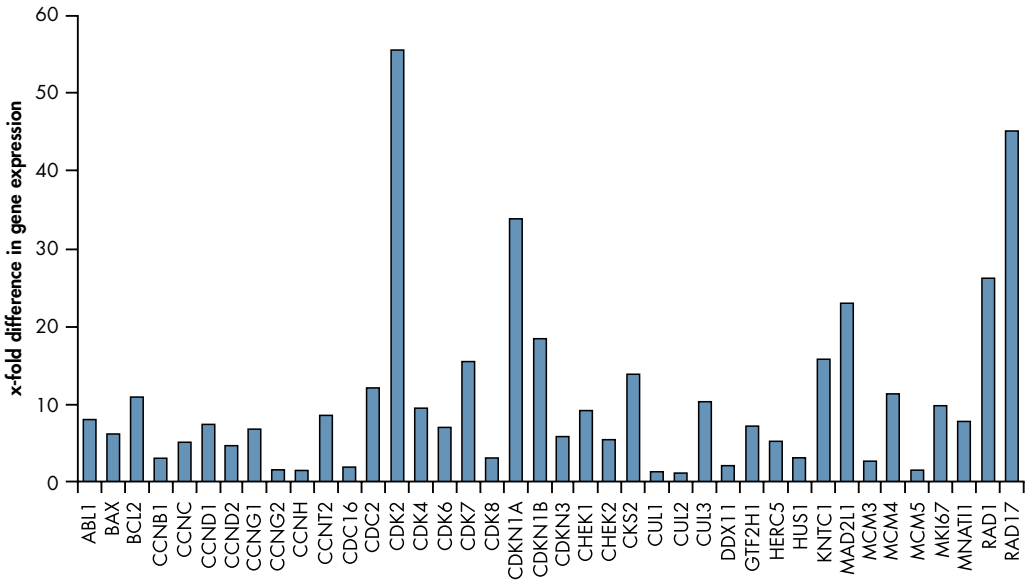


Figure 21. Array analysis following preamplification of cDNA targets. Total RNA was purified from human breast FFPE tissue using the AllPrep DNA/RNA FFPE Kit. RNA was then reverse-transcribed using RT² FFPE PreAMP technology. Gene expression analysis by real-time PCR was performed using the Human Cell Cycle RT² Profiler PCR Array, comparing a tumor sample to a nontumor sample. $\Delta\Delta C_T$ analysis shows the x-fold difference in gene expression of tumor sample compared to nontumor sample.

4.4 Methylation and mutation analysis using Pyrosequencing

Pyrosequencing is a novel technology which allows reliable sequencing of nucleotide sequences as well as sensitive, accurate quantification of genetic variations within the sequences of interest, such as methylation at CpG sites or mutations (Figures 22 and 23). Since DNA molecules in FFPE samples are crosslinked to each other and to other molecules, removal of these crosslinks is required prior to Pyrosequencing analysis. Use of the EpiTect Plus FFPE Bisulfite Kit (Figure 22) or QIAamp DNA FFPE Tissue Kit (Figure 23) to purify genomic DNA from FFPE samples ensures partial reversal of formalin crosslinks (some crosslinks are irreversible), providing suitable DNA template for amplification and subsequent Pyrosequencing analysis.

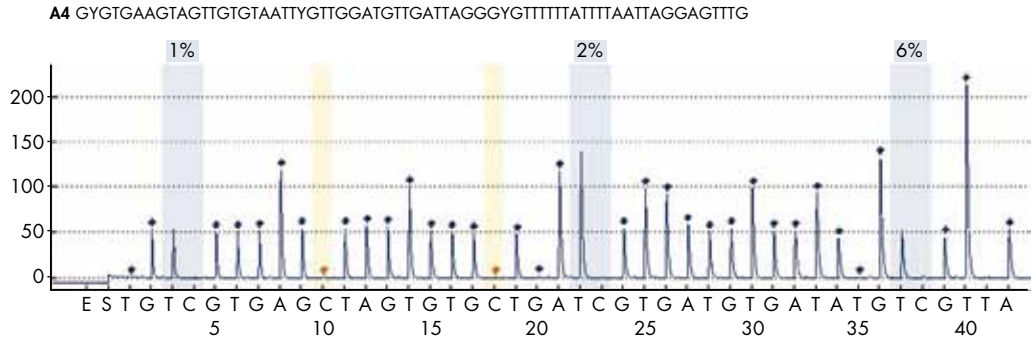


Figure 22. DNA methylation analysis using Pyrosequencing. DNA was purified from rat liver FFPE tissue (10 µm) using the EpiTect Plus FFPE Bisulfite Kit. A segment of the APC gene with 3 CpG sites was amplified using the PyroMark PCR Kit and analyzed by Pyrosequencing using PyroMark Gold Q24 Reagents. The Pyrogram shows reliable long Pyrosequencing reads and sensitive quantification of methylation levels at each CpG site (blue background). Sites highlighted in yellow are control sites demonstrating full bisulfite conversion of the DNA.

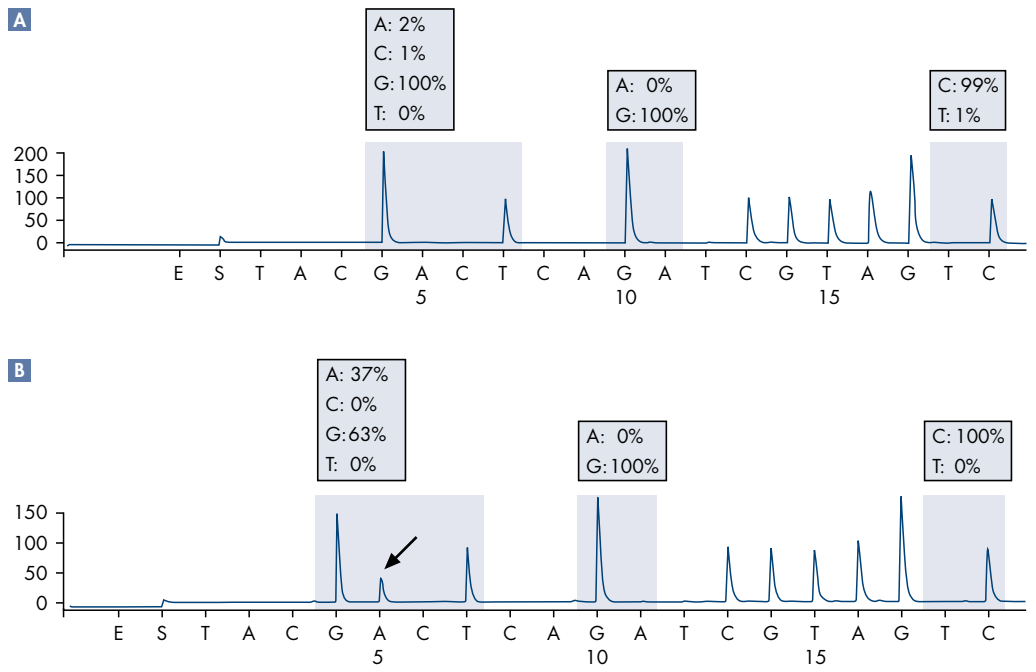


Figure 23. Mutation analysis using Pyrosequencing. DNA was purified from colon cancer FFPE tissue using the QIAamp DNA FFPE Tissue Kit. Pyrosequencing was performed using the KRAS Pyro Kit to identify mutation of the KRAS gene. A Pyrogram trace after analysis of a sample with a normal genotype in codons 12 and 13. B Pyrogram trace after analysis of a sample with a GGT → GAT mutation in base 2 of codon 12 (nucleotide 35, indicated with an arrow).

Ordering Information

Product	Contents	Page	Cat. no.
Deparaffinization Solution	2 x 8 ml	11	19093
QIAamp DNA FFPE Tissue Kit (50)	For 50 DNA preps: Buffers, plasticware, and reagents	12	56404
EZ1 DNA Tissue Kit (48)	For 48 DNA preps: Buffers, plasticware, and reagents	19	953034
REPLI-g FFPE Kit (25)*	For 25 x 50 µl whole genome amplification reactions: Buffers, plasticware, and reagents	13	150243
EpiTect Plus FFPE Bisulfite Kit (48)	For 48 x 25 µl DNA preps with bisulfite conversion	14	59144
RNeasy FFPE Kit (50)	For 50 RNA preps: Buffers, plasticware, and reagents	15	73504
miRNeasy FFPE Kit (50)	For 50 miRNA preps: Buffers, plasticware, and reagents	15	217504
Qproteome FFPE Tissue Kit (20)*	For 20 protein preps: Buffer and plasticware	16	37623
Qproteome FFPE Tissue 2D-PAGE Kit	For 20 protein preps: Buffers, plasticware, and reagents	17	37633
AllPrep DNA/RNA FFPE Kit (50)	For 50 preps: Buffers, plasticware, and reagents	18	80234
QuantiFast Probe Assays (80)*	For 80 x 25 µl reactions; kit included	24	Varies
QuantiFast Probe RT-PCR Plus Kit (80)*	For 80 x 25 µl reactions	23	204482
RT² FFPE PreAMP cDNA Synthesis Kit	For 12 reactions: Buffers and reagents	25	330461
RT² FFPE PreAMP Primer Mixes	Pathway-focused Primer Mixes for all RT² Profiler PCR Arrays	25	Varies
RT² Profiler PCR Arrays	For pathway-focused expression analysis	25	Varies

* Larger sizes available, please inquire.

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Canada ▪ Orders 800-572-9613 ▪ Fax 800-713-5951 ▪ Technical 800-DNA-PREP (800-362-7737)

China ▪ Orders 86-21-3865-3865 ▪ Fax 86-21-3865-3965 ▪ Technical 800-988-0325

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