

Optimized extraction and quantification of RNA from FFPE samples for gene expression analyses

Gene expression analyses on RNA isolated from formalin-fixed, paraffin-embedded (FFPE) tissues are challenging due to chemical modifications of RNA, cross-links of RNA with other molecules, degradation of RNA, and the limited amounts of sample usually available. In this study, researchers at Life Technologies Corporation, the University of Dublin (Ireland), and the University of Bern (Switzerland) addressed the feasibility of obtaining accurate and reproducible gene expression results using FFPE samples that consisted of a wide variety of tissues and block ages. The optimized protocol presented here can be used to reliably and reproducibly quantify mRNA expression levels using RNA isolated from FFPE samples and real-time RT-PCR.

Introduction

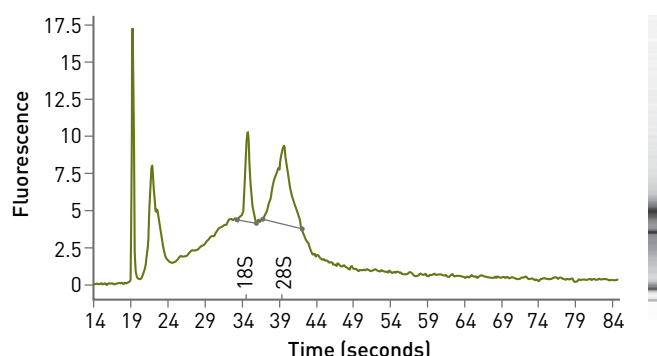
When working with archival material, researchers often need to use RNA from samples that have been stored as FFPE tissue blocks. These FFPE biopsies are a valuable source of information for retrospective research, providing complete historical and clinical information on treatments, procedures, or outcomes.

Although the tissue structure of FFPE samples has been maintained for histological analysis, collateral damage to the nucleic acid incurred through the fixation, embedding, and storage processes can impede measurements of gene expression levels. Nucleic acids are trapped and modified through protein-protein and protein-nucleic acid crosslinks. In addition, RNA is often fragmented and chemically modified to such a degree that it is incompatible with many molecular analysis techniques. The degree of RNA fragmentation that has already occurred in FFPE tissues cannot be reversed, so researchers are faced with the challenge of optimizing RNA gene expression analysis protocols when working with these samples.

Using the following protocol (summarized in Figure 1), RNA from FFPE samples can be reliably and reproducibly assessed by real-time PCR. Real-time PCR methods offer quantitative gene expression results, because they reliably measure steady-state levels of specific RNA sequences regardless of the species (e.g., mRNA, miRNA, snRNA).

1 RNA isolation (FFPE samples)

MagMAX™ FFPE Total Nucleic Acid Isolation Kit or RecoverAll™ Total Nucleic Acid Isolation Kit



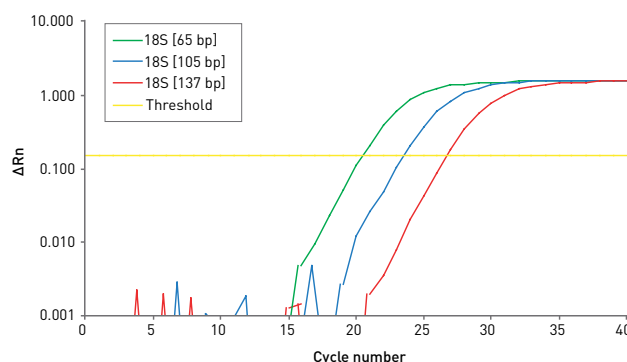
2 Reverse transcription (RT)

SuperScript® VIL0™ or SuperScript® III RT

3 cDNA preamplification

TaqMan® PreAmp Master Mix Kit

- Amplify cDNA targets without introducing bias
- Analyze mRNA from any limited sample (e.g., laser capture microdissections, needle biopsies, and FFPE tissues)
- Stretch as little as 1 ng of cDNA into 200 real-time PCR reactions for gene expression analysis



4 Real-time PCR

TaqMan® Gene Expression Master Mix
TaqMan® Gene Expression Assays
Applied Biosystems® Real-Time PCR Systems

Figure 1. Workflow for gene expression analysis using formalin-fixed, paraffin-embedded (FFPE) samples.

Step 1. RNA isolation from FFPE samples

Isolating RNA from FFPE samples provides several challenges for preserving expression patterns that most closely correlate with the RNA levels present before fixing, embedding, and storage. For example, recovering all mRNA in a quantitative manner can be difficult because some RNA will be cross-linked to other molecules in the FFPE sample. RNA fragmentation, common in FFPE samples, means that RNA isolation protocols must be able to extract all sizes of RNA. In addition, a heating step could be considered as a potential method of reversing formaldehyde-induced modifications of nucleic acids.

Case study: Selecting the best RNA isolation method Comparison of different RNA isolation protocols (quality and yield)

RNA was isolated from a 6-year-old FFPE breast cancer tissue block (10 µm sections) using either the RecoverAll™ Total Nucleic Acid Isolation Kit or one of four other commercial kits. The quality of the resulting RNA was analyzed on an Agilent® 2100 Bioanalyzer™ instrument (Figure 2). As expected (see sidebar *RNA Quality in FFPE Samples*), the RNA integrity is rather low and the broad profiles are characteristic for heavily degraded RNA fragments.

The RecoverAll™ kit has been optimized to recover total RNA including small RNA species. The maximum peak is shifted towards shorter fragment lengths; however, longer fragments are also collected, as is indicated by the overall shape and the shoulder above 200 base pairs in this representative example. In comparison, two other kits (Suppliers 2 and 3) preferentially isolate longer fragments, while two kits (Suppliers 1 and 4) preferentially isolate shorter fragments.

RNA was isolated from breast and lung tumor FFPE samples using the RecoverAll™ kit and four other commercial kits. Yields per 10 µm section were determined by UV absorbance (A_{260}). The RecoverAll™ kit consistently produced the highest yields of RNA (Figure 3).

Conclusions

The RecoverAll™ Total Nucleic Acid Isolation Kit is the optimal solution to extract high yields of RNA of varying sizes, including the small RNA fraction, from FFPE samples. This enables examination of gene expression profiles of both mRNA and miRNA.

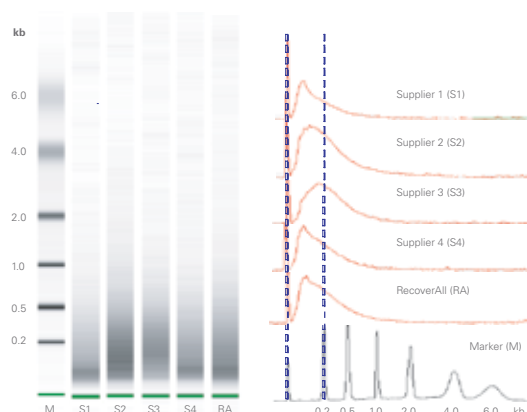


Figure 2. Gel image and electrophoretic traces of RNA from an FFPE sample. RNA preparations from a single FFPE breast cancer tissue block were measured on Agilent® Bioanalyzer™ RNA 6000 Nano chips after utilizing different commercial extraction protocols. Marker = RNA 6000 Ladder [Cat. No. AM7152].

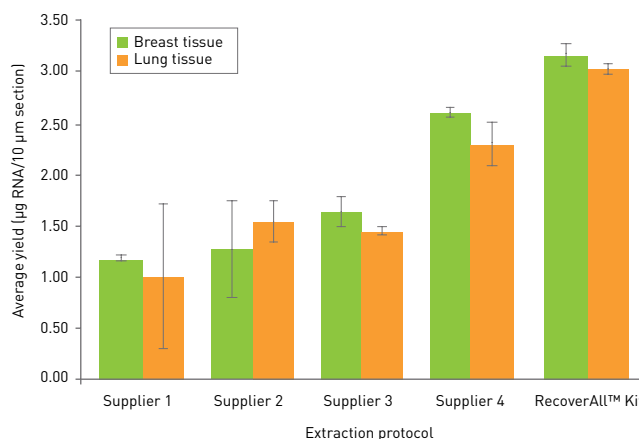


Figure 3. The RecoverAll™ kit provides the highest RNA yield from FFPE tissue. RNA was extracted in duplicate from two distinct 6-year-old FFPE tumor tissues, using five different protocols. The RecoverAll™ kit protocol resulted in the highest yields.

RNA quality in FFPE samples

RNA extracted from FFPE samples is usually heavily degraded, independent of the extraction protocols used. Fragment lengths vary from single bases to several hundreds of base pairs (Figure 2).

Some of the chemical modifications that occur during sample preservation include addition of hydroxymethyl groups and dimerization through methylene bridges [1–2]. The quality and size distribution of the total RNA extracted, as well as an overall quantity of total RNA, can be estimated with adequate instrumentation (e.g., with an Agilent® Bioanalyzer™ instrument) and used to predict the success of subsequent real-time RT-PCR experiments (e.g., by comparing average RNA length and amplicon sizes).

Optional: Effects of heating RNA preparations on FFPE tissues

One of the goals during RNA isolation from FFPE samples is to reduce the amount of chemical modifications that could interfere with downstream enzymatic processes. The classic method used by histone biochemists to reverse formaldehyde-induced modifications of DNA was heating [3]. Heating RNA at 70–95°C for 20 minutes does not negatively impact the quality of the extracted RNA [4], and has successfully been used to treat FFPE samples destined for RNA analyses [5–6].

The RecoverAll™ kit was used to isolate RNA from a thyroid follicular epithelial cancer cell line (Nthy-ori3-1) that was formalin-fixed and paraffin-embedded as a model system for FFPE samples. Real-time PCR assays were used to examine the effects of heating the samples (70°C, 20 min) before column purification of the RNA (Figure 4). The results were also compared to those obtained with RNA isolated from thyroid cancer cells that were not FFPE treated (Fresh Sample, Figure 5).

A decrease in the C_t value was usually observed for identical amounts of input RNA when a heating step was introduced compared to the standard protocol. In this sample series, the average gain in C_t achieved by using the RecoverAll™ kit and the heating step was 2.9 ± 0.6 , which is approximately an 8-fold increase in sensitivity (Figure 5).

Conclusions

The RNA from fresh samples had the lowest C_t value, because the highest quality RNA can be retrieved from fresh samples (Figure 5). Although the observation that C_t values decrease when the sample is heated has been reproduced using a variety of tissues and cell lines (data not shown), in some cases, no improvement in sensitivity was observed. This might be due to different fixation, handling, or storage methods for those samples. However, heating FFPE samples is recommended, since no substantial increase in the C_t value has been observed, indicating that the heating step will not be detrimental to the RNA.

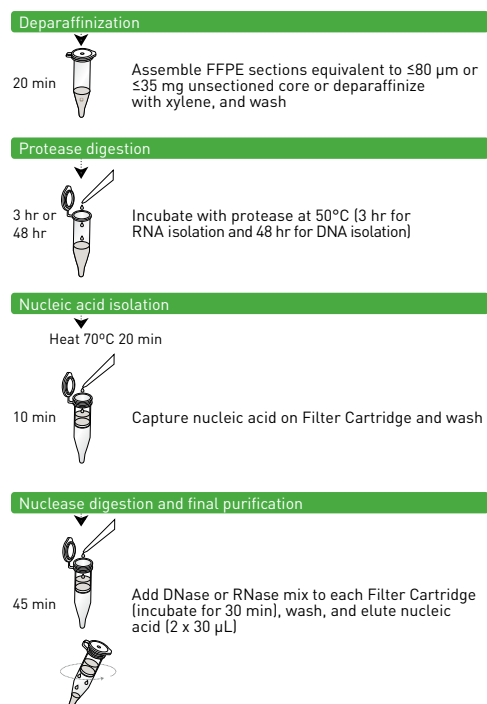


Figure 4. RecoverAll™ kit protocol with additional heating step to reverse some chemical modifications.

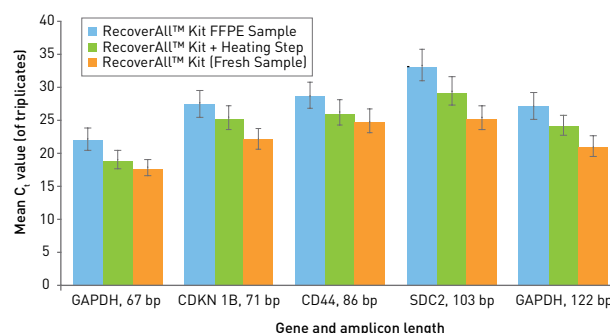


Figure 5. Effect of heating FFPE and fresh samples on RNA amplification. Sample gene expression levels of four different genes (GAPDH was measured with two different TaqMan® Assays) with increasing amplicon length are shown. All RNA was extracted using the RecoverAll™ kit. For one sample (green), the protocol was modified to include a 70°C heating step for 20 min prior to column purification. Real-time PCR was performed using the TaqMan® Gene Expression Master Mix. Error bars indicate the 95% confidence intervals. Data courtesy of J Li and O Sheils, University of Dublin, Trinity College, Dublin, Ireland.

Isolate nucleic acid from FFPE samples: RecoverAll™ Total Nucleic Acid Isolation Kit

The RecoverAll™ Total Nucleic Acid Isolation Kit is designed to extract total nucleic acid from formaldehyde- or paraformaldehyde-fixed, paraffin-embedded tissues. The protease digestion conditions of the RecoverAll™ kit are designed to release a maximal amount of RNA fragments of all sizes, including miRNA, in a relatively short amount of time (3 hr). The recovered RNA is suitable for downstream applications such as reverse transcription, endpoint PCR, real-time PCR, and mutation screening.

Although DNA does not fragment as easily as RNA, the dense nucleohistone matrix appears to be reactive to formaldehyde treatment, so a longer protease digestion time (48 hr) is required to release substantial amounts of DNA. The recovered DNA can be used for PCR and other downstream applications. To see how this kit was used to obtain DNA from FFPE samples for genotyping, visit our website.

Step 2. Reverse transcription

Real-time PCR is a versatile method for the precise quantification of nucleic acids. Its ability to reliably investigate steady-state levels of specific RNA sequences, no matter what the species (mRNA, miRNA, snRNA, etc.), makes real-time PCR (Steps 2 and 4) the method of choice for many quantitative gene expression experiments.

In “two-step” RT-PCR, a reverse transcription step is performed first and the resulting cDNA is then used in separate real-time PCR assays. Therefore, accurate and precise quantification of RNA targets relies upon the performance of the RT step, which means the reverse transcriptase should be highly efficient and have high fidelity.

The greatest flexibility for downstream experiments is usually obtained by using short random oligonucleotides to prime the RT reaction. When working with degraded RNA (e.g., RNA from FFPE samples), we recommend that you test whether random hexamers or amplicon-specific primers give higher reverse transcription yields.

Case study: Selection of reverse transcription kit

Optimal results in downstream assays requires a protocol that maximizes the yield of RT products. The High Capacity cDNA Reverse Transcription Kit uses MultiScribe™ Reverse Transcriptase and random primers to linearly convert up to 2 µg of total RNA to single-stranded cDNA with a 7-log dynamic range (Figure 6A). The efficiency of the RT reaction using this kit was evaluated by real-time PCR (Figure 6B). Three different amounts of input RNA were used to create a standard curve with 11 TaqMan® Gene Expression Assays. The slope of the standard curve indicated equal PCR efficiencies. The slope of the standard curve indicated equal PCR efficiencies.

Conclusions

The High Capacity cDNA Reverse Transcription Kit efficiently and quantitatively produces high quality cDNA for gene expression studies, even with low RNA input amounts. The high efficiency of the MultiScribe™ Reverse Transcriptase in this kit makes it ideal for FFPE samples.

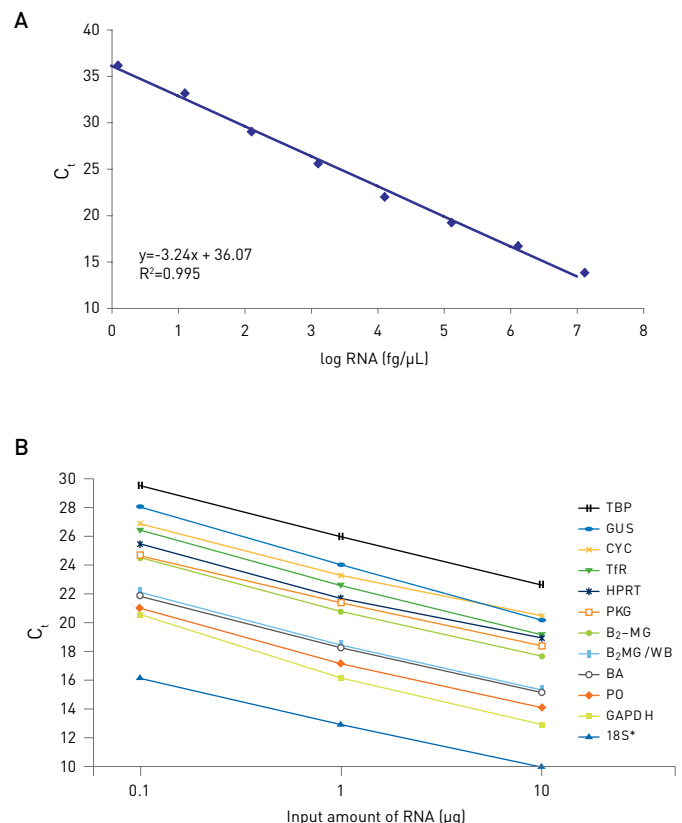


Figure 6. The efficient MultiScribe™ Reverse Transcriptase and the High Capacity cDNA Reverse Transcription Kit produce quality cDNA. (A) A dilution series of Ambion® FirstChoice® Human Brain Reference RNA amplified with the High Capacity cDNA Reverse Transcription Kit on the 7900 Real-Time PCR System demonstrates a wide dynamic range of target detection. **(B)** The expected ΔC_t of -3.3 for each 10-fold increase in input quantity was obtained for 11 different RNA transcripts converted to cDNA from different input quantities of total RNA.

Step 3. cDNA preamplification

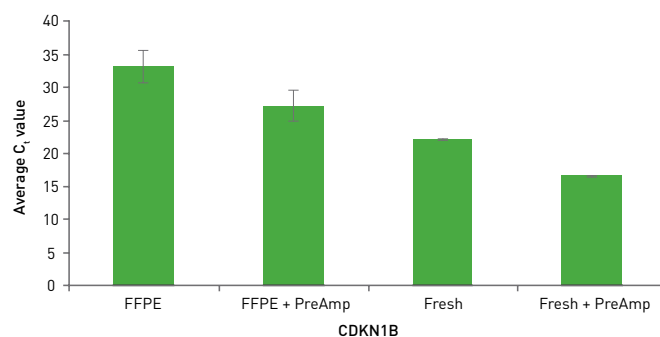
Researchers strive for a high yield RNA extraction procedure to increase the sensitivity of real-time RT-PCR assays using low small samples or low real-time RT-PCR volumes. Often the amount of RNA obtained is insufficient to perform parallel experiments on the same RNA source. cDNA preamplification can be used to address these issues as well as increase data quality. During the preamplification step, cDNA is amplified using methods that do not introduce biases in RNA representation. The amplified product can then be diluted and used in the subsequent real-time PCR experiments.

Case study: Using cDNA preamplification to increase sensitivity of real-time PCR

Real-time PCR was used to evaluate the effects of cDNA preamplification in experiments using the RecoverAll™ kit to extract RNA from matched samples of fresh frozen and laser capture microdissection (LCM), FFPE tissue (Figure 7). The fresh frozen sample was used as a control to show the baseline results before FFPE treatment. Using 10 cycles during the preamplification step should theoretically increase cDNA templates by 1,024 times; however, the 10-fold dilution in the real-time PCR step resulted in an overall 102-fold increase in the cDNA, which is approximately 5.7 cycles (theoretical gain). This value was achieved in most of the experiments using FFPE samples, indicating that little to no bias was introduced during the preamplification step.

Conclusions

The TaqMan® PreAmp Master Mix Kit effectively increases the sensitivity of real-time PCR analysis (with minimal to no bias) of FFPE samples. C_t values are shifted from high ranges down to those where the statistical influences of small copy numbers on C_t values are reduced (in this example, for low-expression genes, from 35 cycles to <30 cycles). Preamplification also enables scientists to perform a greater number of downstream assays on a given sample.



	Average ΔC_t	$\Delta\Delta C_t$
FFPE		
FFPE + PreAmp	6.00 ± 0.04	
Fresh		
Fresh + PreAmp	5.50 ± 0.05	1.5

Figure 7. The TaqMan® PreAmp Master Mix Kit produces artifact-free increases in template for RT-PCR. One tenth of a 10-cycle preamplification reaction was used in a TaqMan® real-time PCR assay. These conditions were predicted to produce a gain in C_t value (ΔC_t) of ~5.7, relative to the original sample (i.e., 10 preamplification cycles increase template 1,024-fold; one tenth of that is 102.4 times the original sample, which is equivalent to a ΔC_t of ~5.7). For most assays, there was little difference between the observed and predicted ΔC_t ; therefore, no preamplification bias was introduced. (TaqMan® Assay for CDKN1B gene, amplicon length = 71 bp; average C_t ± range of two independent extractions.) Data courtesy of J Li and O Sheils, University of Dublin, Trinity College, Dublin, Ireland.

Fast, unbiased RNA preamplification: TaqMan® PreAmp Master Mix Kit

The TaqMan® PreAmp Master Mix Kit amplifies small amounts of cDNA without introducing amplification bias into the sample. Preamplification before real-time PCR enables scientists to examine expression profiles of hundreds of genes in samples with low amounts of starting material (e.g., FFPE tissues, laser capture microdissection (LCM) samples, and needle biopsies) or in high-throughput reactions with small reaction volumes. The preamplification reaction uses a pool of TaqMan® Gene Expression Assays as a source of primers in 10 or 14 amplification cycles to generate approximately 1,000- to 16,000-fold amplification of each gene-specific target. The resulting preamplified reaction is diluted and serves as the starting material for subsequent single-target, real-time PCR with each of the individual TaqMan® Gene Expression Assays represented in the assay pool.

Step 4. Real-time PCR

Compared to the two other commonly used techniques for quantifying mRNA levels (Northern blot analysis and ribonuclease protection assay), real-time PCR can be used to quantify mRNA levels from much smaller RNA input amounts and over a broader dynamic range. The development of novel chemistries and instrumentation platforms enabling detection of PCR products in real-time has led to the widespread adoption of real-time PCR as the method of choice for quantifying changes in gene expression. Reaction products are measured for each sample in every amplification cycle, resulting in a broad 9-log dynamic range of detection.

Primer design, master mixes, and real-time PCR systems

FFPE treatment and storage of tissue samples leads to RNA that is randomly damaged across its length. For amplifications from these samples there is a direct correlation between target amplicon size and the number of intact target templates present. Therefore, shorter amplicons will perform better [7–9]. We recommend using primers that generate amplicons of less than 150 bp.

Case study: Optimizing real-time PCR assays

Primer design: Amplicon size

Antonov and colleagues investigated the influence of amplicon size on real-time PCR results using artificially degraded (alkaline treatment) RNA from a breast cancer cell line [8]. This report showed that mRNA length had less of an effect on performance (sensitivity or efficiency) when RNA molecules were less degraded (i.e., longer) and the amplicons used during the real-time PCR step were shorter (in agreement with findings of Godfrey et al [9]).

Here, the effects of amplicon length when using artificially degraded RNA from a breast cancer cell line were tested by real-time PCR with three custom TaqMan® Gene Expression Assays. Different amplicon lengths were generated by varying the location of the reverse primer (the probe and forward primer were the same for all assays) (Figure 8). Target detection increased with decreasing amplicon length, as observed by the lower C_t values for shorter amplicons.

Although the amplicon locations did not overlap, a similar effect as also seen in Figure 5 for RNA from the FFPE samples. The C_t value for the shorter GAPDH amplicon was lower than that for the longer GAPDH amplicon. However, the difference in C_t values could also be partly due to differences in primer/amplicon design, as primers and amplicon spanned a different exon–exon boundary.

Master mixes and real-time PCR systems

In addition to primer design (for reverse transcription and PCR), the choice of the master mix and real-time PCR systems can also influence sensitivity of detection. Applied Biosystems scientists have optimized several master mixes for various applications and recommend the TaqMan® Gene Expression Master Mix for analysis of FFPE samples. This master mix contains all components necessary for PCR assays, except cDNA and primer/probes. In addition, Applied Biosystems® real-time PCR systems have been

acknowledged as the gold standard in real-time PCR, whether you need high-throughput capability (7900HT Fast Real-Time System) or you are just getting started (StepOne™ Real-Time PCR System).

Conclusions

When designing real-time RT-PCR experiments for FFPE samples, use TaqMan® Gene Expression Assays with the shortest amplicons, the TaqMan® Gene Expression Master Mix, and Applied Biosystems Real-Time PCR Systems (see sidebar *TaqMan® Gene Expression Master Mix and Assays*).

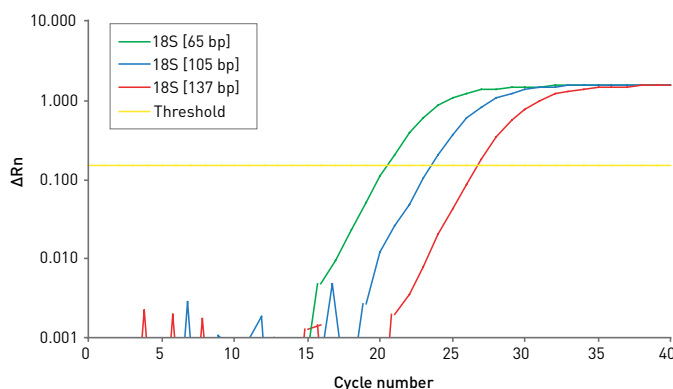


Figure 8. Effects of amplicon size on real-time RT-PCR using compromised input RNA. A shift to a higher C_t was observed for artificially degraded (alkaline-treated) RNA isolated from a ZR75 breast cancer cell line (reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit, Real-time PCR was performed using TaqMan® Universal PCR Master Mix and custom TaqMan® MGB probes). Data courtesy of A Oberli, A Baltzer, and R Jaggi, University of Bern, Switzerland.

TaqMan® Gene Expression Master Mix and Assays

TaqMan® Gene Expression Master Mix

TaqMan® Gene Expression Master Mix provides a real-time PCR mix that may be used with any appropriately designed primer and probe set to detect any DNA target (including cDNA, genomic DNA, and plasmid DNA). The mix is optimized for TaqMan® assays and contains AmpliTaq Gold® DNA Polymerase UP (Ultra Pure); uracil-DNA glycosylase (UDG; to prevent reamplification of carry-over PCR products); deoxyribonucleotide triphosphates (dNTPs) with deoxyuridine triphosphate (dUTP); and ROX™ passive reference dye (to serve as an internal reference for reporter-dye signal normalization). This is the master mix recommended for use with RNA isolated from FFPE samples.

TaqMan® Gene Expression Assays

TaqMan® Gene Expression Assays are a comprehensive collection of more than 700,000 predesigned primer and probe sets that let researchers quickly and easily perform quantitative gene expression studies on human, mouse, rat, canine, Rhesus macaque, *C. elegans*, *Drosophila*, and *Arabidopsis* genes. These assays provide the exquisite sensitivity, linearity, and dynamic range that you've come to expect from TaqMan® chemistry, and all perform under universal thermal cycling conditions.

TaqMan® Gene Expression Assays utilize Minor Groove Binding (MGB) probes to increase sequence specificity, and the small amplicon sizes (often well below 100 base pairs) make them an ideal solution for real-time RT-PCR using degraded RNA from FFPE samples. The amplicon length for each TaqMan® Gene Expression Assay can be found on the Applied Biosystems website (Figure 9).

Find TaqMan® Gene Expression Assays for your genes of interest at www.allgenes.com.

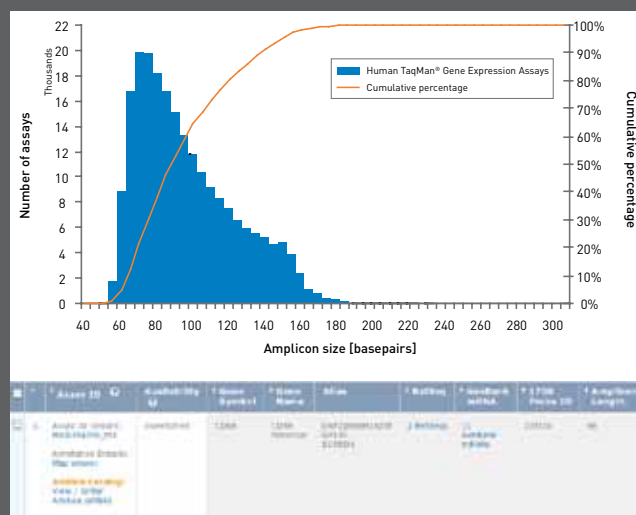


Figure 9. Distribution of amplicon sizes for TaqMan® Gene Expression Assays. Amplicon sizes targeting the human genome and screenshot of the TaqMan® Gene Expression Assay search results page on our website showing the amplicon size for the CD68 gene assay.

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Workflow: gene expression analysis using FFPE samples

FFPE methylation sequencing workflow			
1. RNA isolation	2. Reverse transcription	3. cDNA preamplification	2. Reverse transcription
PRODUCTS <ul style="list-style-type: none">RecoverAll™ Total Nucleic Acid Isolation Kit or MagMAX™ FFPE Total Nucleic Acid Purification Kit	PRODUCTS <ul style="list-style-type: none">SuperScript® III or SuperScript® VILO™ RTHigh Capacity cDNA Reverse Transcription Kit	PRODUCTS <ul style="list-style-type: none">TaqMan® PreAmp Master Mix	PRODUCTS <ul style="list-style-type: none">TaqMan® Gene Expression Master MixTaqMan® Gene Expression Assays

Green: Ambion® kits and reagents

Blue: Applied Biosystems® reagents and instruments

Ordering information

Product	Quantity	Cat. No.
Nucleic acid isolation		
RecoverAll™ Total Nucleic Acid Isolation Kit	40 purifications	AM1975
MagMAX™ FFPE Total Nucleic Acid Isolation Kit	96 preps	4463365
MagMAX™ FFPE DNA Isolation Kit	96 preps	4463578
Reverse transcription		
High Capacity cDNA Reverse Transcription Kit	200 rxns	4368814
cDNA reamplification		
TaqMan® PreAmp Master Mix Kit	40 rxns	4384267
GeneAmp® PCR System 9700 Thermal Cycler	1 instrument	Inquire
9800 Fast Thermal Cycler	1 instrument	Inquire
2720 Thermal Cycler	1 instrument	Inquire
Veriti® Thermal Cycler	1 instrument	Inquire
Real-time PCR		
TaqMan® Gene Expression Master Mix	40 rxns	4370048
	200 rxns	4369016
TaqMan® Gene Expression Assays—Inventoried	250 rxns	4331182

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