

## CHAPTER 9

# *Procedures for Quality Control of RNA Samples for Use in Quantitative Reverse Transcription PCR*

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## 9.1 Introduction

The quality of any scientific data is directly proportional to that of the original starting samples, or simply 'garbage in, garbage out'. In most circumstances it is logical to work with the highest quality material possible. However, for some experiments the highest quality possible is still a serious compromise from perfection. The degree to which the standard of input material influences final quantitative reverse transcription polymerase chain reaction (qRT-PCR) data and, potentially, the resulting scientific conclusion, is outlined in this chapter.

## 9.2 RNA Extraction Approaches

In order to have the best possible chance of extracting high-quality RNA, tissue and cell samples should be extracted from the source and RNase activity prevented as quickly as possible.

### 9.2.1 Freezing

Solid tissue biopsies need to be stabilised immediately at source with subsequent RNA extraction procedures carried out when required. This can be achieved by snap-freezing in liquid nitrogen. For these samples, labelling

and cataloguing must be rigorous to ensure rapid and accurate retrieval and that tube markings are not removed during freezing. Automated storage and retrieval systems have revolutionised the whole process of sample tracking.

### 9.2.2 Sulfate

An alternative to freezing is to immerse tissues into aqueous sulfate salt solutions (such as ammonium sulfate) at controlled pH and ambient temperature. This treatment results in precipitation of RNases and other solubilised proteins and protects tissue RNA. Tissue samples should be prepared as slices less than 0.5 cm, preferably 2 mm.<sup>1</sup> A larger relative surface area facilitates diffusion of the solution into the tissue. Treated tissues can be stored at  $-60^{\circ}\text{C}$  prior to processing using standard RNA preparation techniques.<sup>2</sup> This technique forms the basis of the commercially available *RNAlater*<sup>®</sup> solution (Ambion Inc., Applied Biosystems, USA). Small organs such as rat livers or kidneys can be immersed whole in solution, small sections of tissue less than 0.5 cm thick should be stored in 5 volumes *RNAlater*<sup>®</sup>. Cell culture pellets can be re-suspended in a minimal volume of phosphate buffered saline (PBS) and then 5–10 volumes *RNAlater*<sup>®</sup> added. Samples can be stored in *RNAlater*<sup>®</sup> at ambient temperature for up to 1 week, or long term at  $-20^{\circ}\text{C}$ . *RNAlater-ICE*<sup>®</sup> (Ambion Inc., Applied Biosystems, USA) has been developed to aid tissue processing of previously frozen material. These samples are then processed using conventional column or phenol based systems such as TRI<sup>®</sup> Reagent (Sigma Aldrich, USA).

### 9.2.3 Guanidinium Isothiocyanate

It is preferable to harvest adherent cultured cells directly in lysis buffer containing guanidinium isothiocyanate. This process enables maintenance of representative cellular messages because it ensures rapid inactivation of RNases that are released during trypsin treatment and can subsequently initiate mRNA degradation. Similarly, small tissue sections can be homogenised directly in guanidinium isothiocyanate lysis buffer.

### 9.2.4 Phenol

Alternatively cells or tissue can be disrupted in TRI<sup>®</sup> Reagent. These homogenates can also be stored at  $-80^{\circ}\text{C}$  until RNA purification is required. RNA extracted using these phenol-based protocols results in a high yield of nucleic acid, but care must be exercised to ensure high levels of purity.

### 9.2.5 Additional Purification

In some cases it is appropriate to perform a subsequent column purification step and DNase I digestion to ensure removal of protein and genomic DNA (gDNA) contamination. Column-based purification procedures in kit format

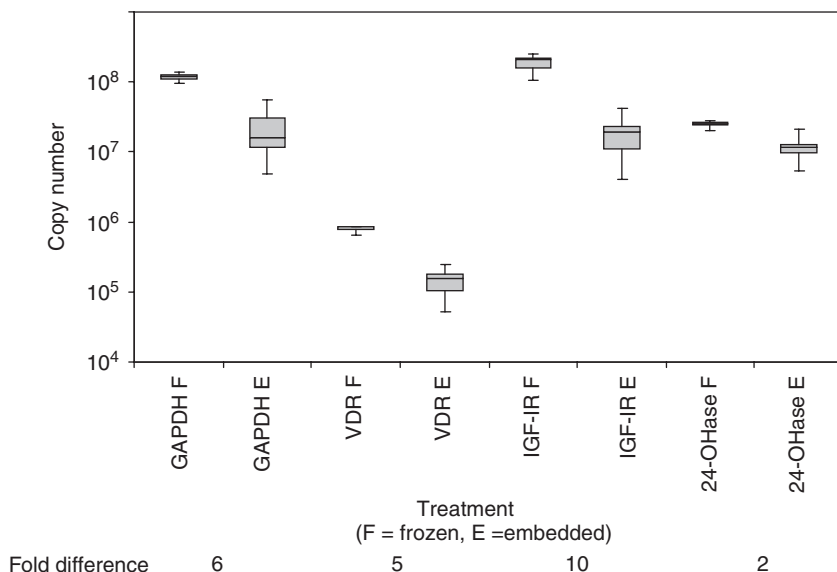
usually produce pure RNA samples. In most cases a gDNA removal procedure is incorporated into the protocol. Performing this reaction *via* a column ensures that residual gDNA or any DNase I activity does not remain in the sample.

### 9.2.6 Extraction from Archival Tissue Samples

Archived formalin fixed, paraffin embedded tissue (FFPE) samples have been explored as a rich source of RNA from samples with complete histological profiles. These samples offer the potential to investigate a number of disorders because they are usually accompanied by detailed medical histories and clinical outcomes.<sup>3</sup> RNA from fixed tissues is usually more difficult to extract due to cross-linking to proteins<sup>4</sup> and the fixation and storage process often results in RNA degradation.<sup>5</sup> The fixing process also results in mono-methylol modifications on all bases, which results in inhibition of subsequent reverse transcription reactions.

It is for these reasons that RNA extracted from formalin fixed material is invariably low quality and can produce results that deviate from those derived from fresh tissues. In an investigation into the potential influence of sample processing and storage, tissue sections were divided and sections either frozen or formalin fixed. RNA was extracted from each section and the copy number of specific mRNA targets was determined using gene-specific reverse transcription and reference to a calibration curve constructed from an artificial oligonucleotide. The hypothesis was that the proportion of transcripts detected in fresh tissue relative to formalin fixed tissue would remain constant if the fixation procedure affected all tissues equally, indicating that fixed material could be used as a reliable source of RNA for qRT-PCR determination of gene quantification. The initial observation was that there was an increase in the variability of quantities detected in the replicate tissues sections after formalin fixation when compared to the reproducibility in quantities measured in samples after freezing. There was a six-fold relative difference in the quantity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) extracted from frozen and fixed tissues. However, this difference was not consistent for other transcript quantities; there was a five-fold difference in the quantity of vitamin D receptor (VDR) between the two tissue treatments, a ten-fold difference in insulin-like growth factor I receptor (IGF-IR) and yet only a two-fold difference in 24-O Hydroxylase (24-OHase). The variability in the observed differences demonstrates that sample freezing and formalin fixation result in inconsistencies in transcript quantification, potentially resulting in different biological conclusions<sup>6</sup> (Figure 9.1).

In an extensive study of the factors influencing qRT-PCR of extracts from FFPE material, Godfrey *et al.*<sup>7</sup> demonstrated that the highest quality RNA was produced after two sequential TRIzol<sup>®</sup> (Invitrogen, UK) extractions, targets were more efficiently detected with amplicons < 130 bp and careful optimisation of RT conditions were required. Despite optimisation improving the data from fixed tissue the authors note that detection of targets is less efficient from



**Figure 9.1** Graph showing the amount of RNA quantified from frozen (F) formalin fixed embedded (E) tissue samples. The fold difference in RNA detected for four transcripts is shown; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), vitamin D receptor (VDR), insulin-like growth factor I receptor (IGF-IR), 24-O Hydroxylase (24-OHase).

fixed material and that the effect on different mRNA species, and even different fragments of the same mRNA, is variable<sup>8</sup> (also shown in Figure 9.1). Interestingly these authors report that pre-fixation time had the least effect on mRNA quantification but Macabeo-Ong *et al.*<sup>9</sup> report that prolonged formalin fixation had a detrimental effect on qRT-PCR. A further technical problem is highlighted by Williams *et al.*,<sup>10</sup> who demonstrated that in RNA extracted from FFPE tissue, as many as 1:500 bases are mutated. These base changes are either C to T or G to A transitions. These data indicate that quantification of mRNA from formalin fixed tissue must be carried out with great care and with the knowledge that relative transcript quantities may not be accurate.

A relatively new detection approach, the QuantiGene<sup>®</sup> branched DNA detection method (Panomics Inc., USA), may be more appropriate than qRT-PCR for the detection of damaged and chemically modified material.<sup>11,12</sup>

### 9.3 RNA Quality

Since tissue storage and treatment and RNA extraction procedures are so variable it is imperative that a reliable protocol for analysis of sample quality and quantity is defined. A full description of an RNA sample requires a statement regarding quality and a measure of quantity. RNA quality is a factor of both the purity of the sample and the degradation status of the RNA

molecules. When the sample is to be used for measurements of transcript quantity one relevant measurement is a determination of whether the mRNA molecules are degraded.

Traditionally, analysis of RNA quality was by gel electrophoresis and analysis of the ratio of the quantities of the ribosomal RNA molecules. Using the ratio of the ribosomal fragments is unreliable because it relies on transcript independent molecules to infer the mRNA status.

### 9.3.1 RNA Integrity Number

In a recent report, Schroeder *et al.*<sup>13</sup> suggested that it is possible to calculate a more objective measure of RNA quality by measuring characteristics of the electropherogram generated by the Agilent 2100 Bioanalyzer, including the fraction of the area in the region of 18S and 28S rRNA, the height of the 28S peak, the presence or absence of RNA degradation products, the fast area ratio and marker height. These features were used to calculate and assign an RNA Integrity Number (RIN) to each RNA sample. RIN values range from 1 for completely degraded samples to a value of 10 for completely intact RNA. However, in an elegant study to investigate the influence of RNA integrity on qRT-PCR assay performance, Fleige and Pfaffl<sup>14</sup> reach a different conclusion. The authors extracted RNA samples from numerous bovine tissue types, subjected them to controlled degradation and analysed them using the Agilent 2100 Bioanalyzer. The samples had RINs between 10, which were apparently intact, to 4 with almost no evidence of rRNA bands. The quantity of individual transcripts in each of these samples was then determined using qRT-PCR assays. In some tissues the quantity of the measured transcripts was independent of RIN whereas in others there was a linear relationship and in still others a threshold response. Critically, the relationship between transcript quantity and RIN was different for different tissues and different transcripts and there was not a predictable relationship between these factors. The authors conclude that moderately degraded RNA samples can be reliably analysed and quantified using short amplicons (<250 bp) and expression is normalised against an internal reference and recommend that a RIN of at least 8 is required to assume that RNA is high quality. Similarly, in an evaluation of the stability of reference gene transcripts in experimental samples Pérez-Novo *et al.*<sup>15</sup> conclude that 'it is inappropriate to compare intact and degraded samples'. The discrepancy between the initial report describing the RIN algorithm and these evaluations of the correlation of RIN to transcript quantification<sup>14,15</sup> could be due to the relatively poor correlation coefficient (0.52) between RIN and expression values of the reference genes reported by the authors advocating the use of RINs.<sup>13</sup>

In the absence of an alternative reliable measure of mRNA integrity, the use of a 3':5' assay using GAPDH as the target sequence has been proposed.<sup>16</sup>

The data obtained are independent of ribosomal RNA integrity, provide a reasonable measure of the degradation of the transcripts of interest and are

modelled on the standard approach adopted by microarray users and long-accepted conventional techniques applied to end-point PCR assays.<sup>17</sup> The 3':5' assay measures the integrity of the ubiquitously expressed mRNA specified by the GAPDH gene, which in this example is taken as representative of the integrity of all mRNAs in an RNA sample. However, since different mRNAs degrade at different rates, this may not always be the case and it may be necessary to design similar assays for specific targets. The RT reaction of the GAPDH mRNA is primed using oligo-dT, and a separate multiplex PCR assay is used to quantify the levels of three target amplicons. These are spatially separated with one towards the 5' end, the second towards the centre and the third towards the 3' end of the mRNA sequence. The ratio of amplicons reflects the relative success of the oligo-dT primed RT to proceed along the entire length of the transcript. This is prematurely terminated when mRNA is degraded. Consequently, a 3':5' ratio of around 1 indicates high integrity, whereas anything greater than 5 suggests degradation. The assay is designed as a triplex assay using TaqMan™ chemistry such that each amplicon is detected by a target-specific, differentially labelled probe. An example of the use of the 3':5' assay to evaluate RNA samples is shown in Figure 9.2.

The 3':5' assay is particularly applicable for analysis of precious samples when little RNA is available. An example of analysing RNA extracted from FFPE tissue is shown in Figure 9.2C. There are at least 4 Cts difference between the detection of each of the GAPDH assays indicating that this RNA is seriously degraded.

### 9.3.2 Spectrophotometric Measurement

$A_{260}/A_{280}$  measurements are often made in an attempt to assess the quality of nucleic acid samples. These measurements are based upon the ratio between the absorbance of nucleic acid at  $A_{260}$  and protein and indicate absorbance of protein and phenol at  $A_{280}$ . A ratio below 1.8 generally indicates the presence of substances absorbing at  $A_{280}$  and usually the sample is considered to contain contamination. It is clear that this is not a reliable measure of sample quality since it is limited in the range of substances detected and does not reveal degradation state.

### 9.3.3 Presence of Inhibitors

Inhibitory components frequently found in biological samples can result in a significant reduction in the sensitivity and kinetics of qPCR.<sup>18–23</sup> The inhibiting agents may be reagents used during nucleic acid extraction or co-purified components from the biological sample, for example bile salts, urea, heme, heparin or IgG. The potential inaccuracies occur when an external calibration curve is used to calculate the number of transcripts in test samples. Invariably the material used to produce the calibration curve is biologically distinct from the test material, which is more likely to contain inhibitors. This leads to an underestimation of the mRNA levels in the test samples.<sup>24</sup> As discussed

previously, the increasing interest in extracting nucleic acids from FFPE archival material will undoubtedly lead to an exacerbation of this problem.

The most common procedure used to account for any differences in PCR efficiencies between samples is to amplify a reference gene in parallel to the reporter gene and relate their quantification. However, this approach assumes that the two assays are inhibited to the same degree. In an attempt to demonstrate the effect of a contaminating agent in an RNA sample, EDTA was added to purified RNA samples to a final concentration of 125 mM. This sample was also included in the GAPDH 3':5' assay analysis described previously as an assay for detection of degraded RNA (Figure 9.3). A clear shift to higher Ct was observed for both the 3' and 5' GAPDH assays (relative to the

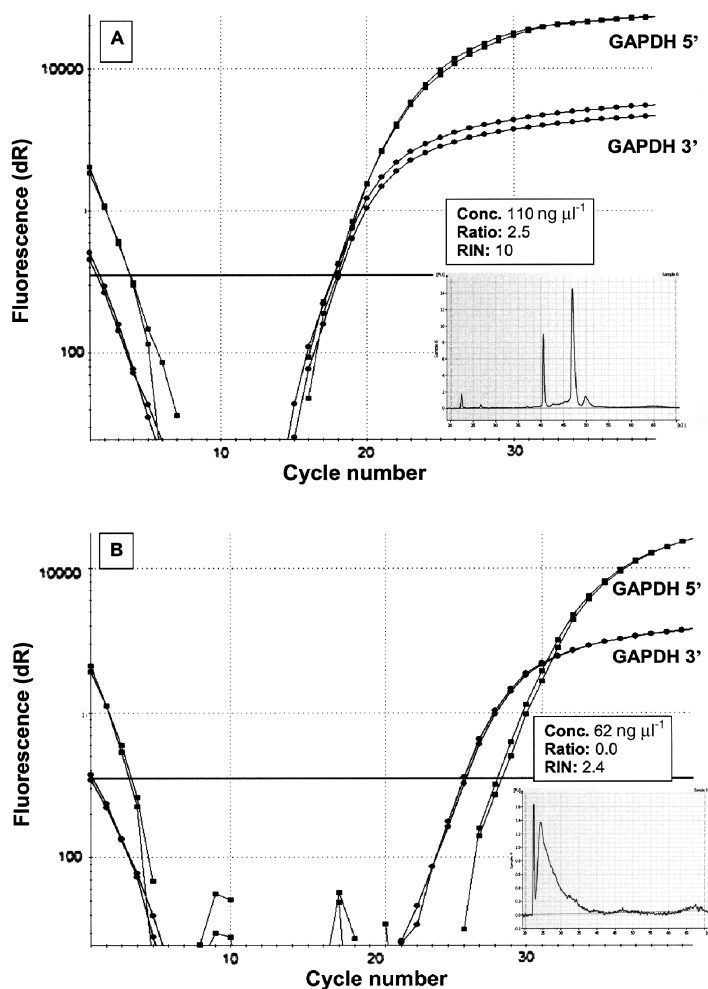
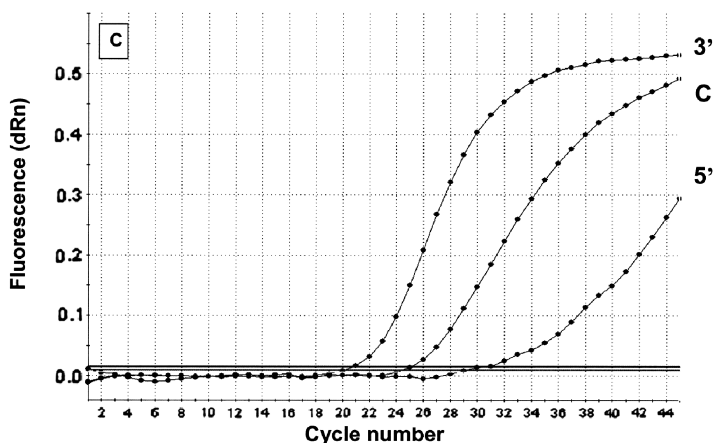


Figure 9.2 (Continued)

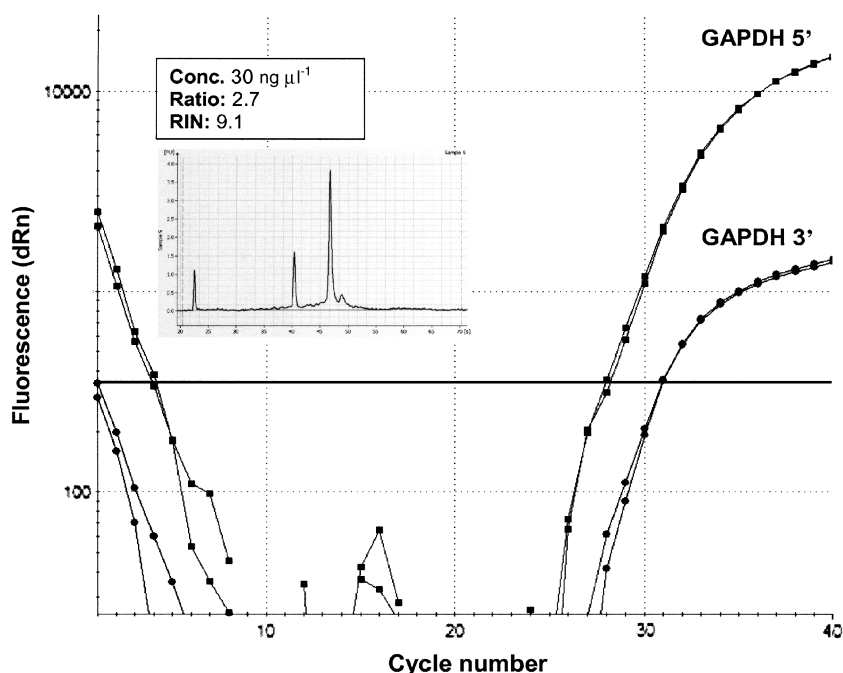


**Figure 9.2** GAPDH mRNA quantified from oligo dT primed cDNA using three individual qPCR assays targeting 5', centre and 3' regions. (A) This sample has an Agilent 2100 Bioanalyzer RIN of 10 and equal concentrations of 5' and 3' assay target sequences. (B) The second sample appears to be seriously degraded, RIN 2.4, and has an apparently lower concentration. The 3' GAPDH assay detects a higher concentration of target with Ct 24 than the 5' with Ct 27 confirming degradation of this sample. Since the second sample was produced from the first it is important to note that the shift in Ct from 18 to 24 for the 3' assay is indicative of the degree of degradation. (C) RNA extracted from FFPE tissue showing differences in the quantities of 5', centre and 3' sequences indicating that this RNA is seriously degraded.

pure RNA sample, Figure 9.2A). In the presence of the inhibitor both reactions are inhibited but the effect on the 5' reaction is more pronounced than on the 3'. Since it can be assumed that reverse transcription of the 3' site must precede that of the 5' site, the higher yield of the 5' target is due to greater sensitivity of the 3' assay to the effect of EDTA inhibition. This is a single example that clearly indicates that qRT-PCR assays may be differentially affected by inhibitors. This demonstrates clearly that it is inappropriate to assume that the effect of inhibition is equal for all qPCR assays. Therefore, the presence of inhibitors cannot be cancelled by reference to a second target amplification or normalisation to a reference gene.

Various methods can be used to assess the presence of inhibitors within biological samples. The efficiency of the PCR in a test sample can be assessed by serial dilution of the sample,<sup>25</sup> although this is practically impossible for every sample of a high-throughput study or when using very small amounts of precious RNA. Alternatively, there are various algorithms that provide an estimate of PCR efficiency from analysis of amplification curves.<sup>26–28</sup> Internal amplification controls (IAC) that co-purify and co-amplify with the target nucleic acid can be used to detect inhibitors as well as indicate template loss during processing.<sup>29</sup> Another approach utilises a whole bacterial genome to detect inhibition from clinical samples.<sup>30</sup>

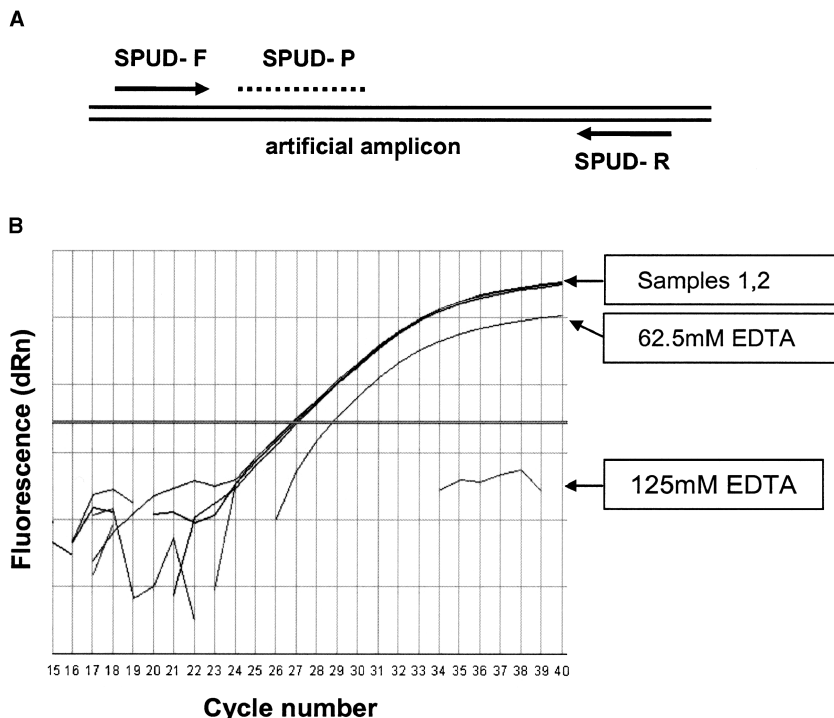




**Figure 9.3** Illustration of the effect of inhibitors. EDTA was added to an RNA sample to a final concentration of 125 mM and this was assessed using the GAPDH 3':5' assay for detection of degraded RNA (see Figure 9.2). A clear shift to higher Ct was observed for both the 3' and 5' GAPDH assays with a more pronounced shift of the 5' reaction.

Nolan *et al.*<sup>31</sup> describe the use of a universal qPCR reference assay, known as SPUD, to identify inhibitors of the reverse transcription or PCR steps by recording the relative Cts characteristic of a defined number of copies of a sense-strand amplicon. An artificial amplicon (SPUD-A) is amplified using two primers (SPUD-F) and (SPUD-R) and the products are detected using a TaqMan<sup>™</sup> probe (SPUD-P) (Figure 9.4A). In the presence of water, a Ct is recorded that is characteristic of an uninhibited reaction (dependent on amplicon copies used and technical variabilities). Alongside this reaction, which contains only the SPUD amplicon, reactions are run which contain exactly the same components (SPUD-A, SPUD primers and SPUD probe) together with the unknown test sample (RNA or DNA). Potential inhibitors in the test sample will result in a shift to higher Ct for these reactions when compared to those where the test sample is absent.

Conventional  $A_{260}/A_{280}$  measurement does not detect the presence of high concentrations of EDTA that are clearly detrimental to qPCR amplification in an assay-specific manner (data not shown). Interestingly, electrophoretic traces of these samples result in a comparatively low estimate of RNA concentration in the presence of EDTA although these were derived from the



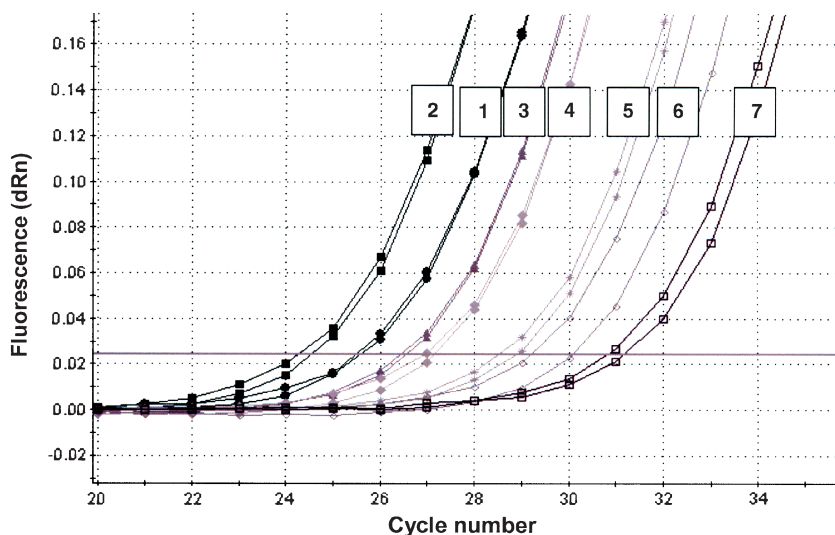
**Figure 9.4** Use of the SPUD universal qPCR reference assay. (A) In the presence of water, a  $C_t$  is recorded that is characteristic of an uninhibited SPUD reaction. (B) Amplification of the SPUD amplicon in the presence of the intact and degraded samples (samples 1 and 2 respectively) does not affect the assay whereas EDTA at 62.5 mM caused a 2  $C_t$  shift and EDTA at the higher concentration prevented all amplification in the SPUD assay.

purified samples and known to be of equal concentration ( $110 \text{ ng} \mu\text{l}^{-1}$ ). It is worthy of note that EDTA suppressed the fluorescence reading by both the Agilent 2100 Bioanalyzer and the Bio-Rad Experion systems. RNA samples containing EDTA at final concentrations of 125 mM and 62.5 mM were included in the SPUD assay alongside purified RNA and purified, degraded RNA samples (Figure 9.4B). Amplification of the SPUD amplicon in the presence of the purified RNA samples and the degraded sample resulted in exactly the same  $C_t$  (27). In the presence of EDTA at the lower concentration the  $C_t$  is shifted by 2  $C_t$ s and at the higher concentration no amplification is detected. In this example the EDTA is sufficient to prevent any amplification of the SPUD amplicon. This demonstrated that the SPUD assay is a useful tool for identification of inhibitors in samples and the application of the technique to screening samples extracted from FFPE sections is shown by Nolan *et al.*<sup>31</sup> Novak and Huggett have also demonstrated that the system can be used to identify false negative results due to inhibition of the test PCR.<sup>32</sup>

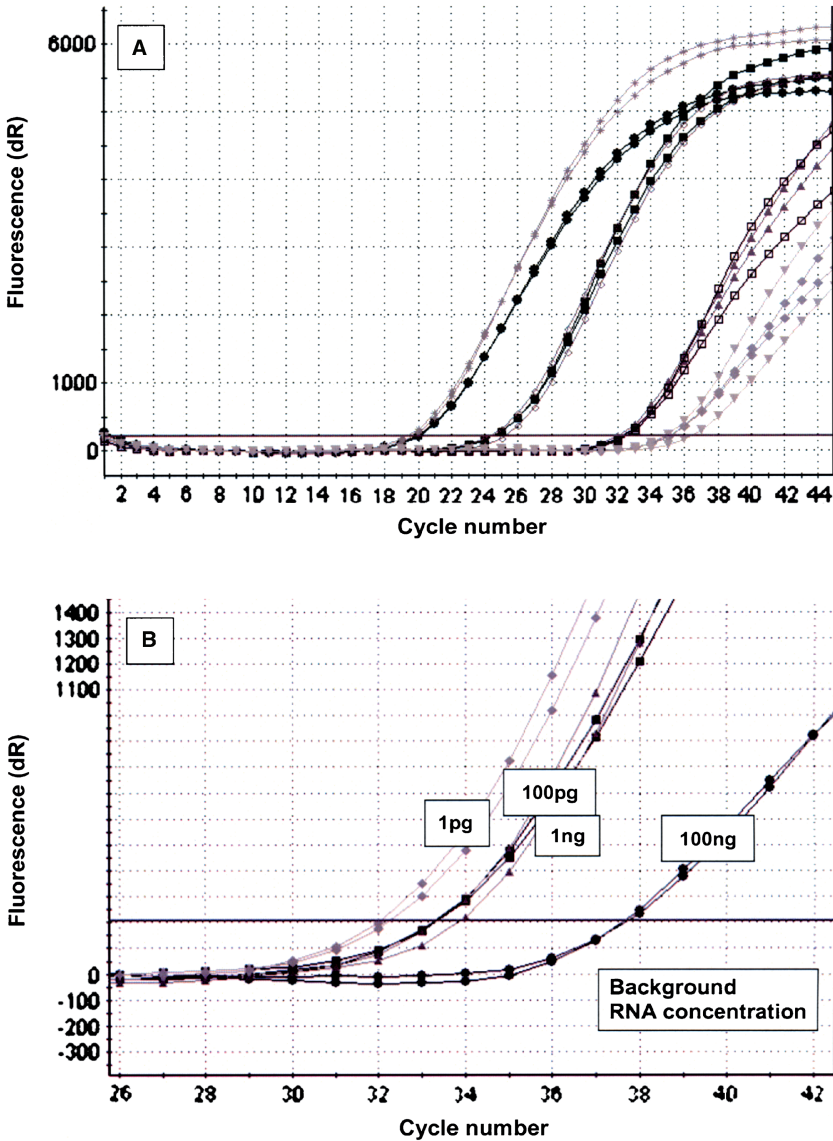
## 9.4 RNA Quantification

### 9.4.1 Significance of Quantification

Many downstream molecular biology assays that use RNA are sensitive to template concentration. This can be demonstrated by reverse transcription of a serial dilution of RNA and quantification of specific cDNA targets. An example of this phenomenon is illustrated in Figure 9.5. In this experiment, cDNA was produced from a five-fold serial dilution of total RNA using StrataScript<sup>®</sup> reverse transcriptase (Stratagene, UK) and random nonamer primers. The quantity of a number of target genes was determined and it is clear that there was not a linear relationship between the initial concentration of RNA and the level of the specific cDNA yield. At the most extreme there is an inverse relationship between the most concentrated RNA sample and the cDNA yield (the first two amplification plots are 'reversed') indicating that high concentrations could inhibit reverse transcription. In a further study RNA was diluted 100-fold, cDNA produced as described previously and the quantity of  $\beta$ actin was determined (Figure 9.6A). The cDNA synthesis was replicated using the same RNA dilution series and duplicate qPCR reactions were run from each independent RT series. As before there was not a linear relationship between the initial RNA and cDNA yield but the yield of  $\beta$ actin was reproducible. A constant number ( $10^4$ ) of copies of a specific target sequence was added to each RNA dilution sample and cDNA made from the mixture. The number of copies of the spike sequence in each sample was then determined



**Figure 9.5** qRT-PCR quantification of  $\beta$ actin from cDNA produced from five-fold serial dilution (1–7) of total RNA. The lowest Ct was recorded from the sample containing the second highest concentration of RNA demonstrating that high concentration of RNA could inhibit reverse transcription.



**Figure 9.6** Effect of RNA concentration. (A) cDNA was produced from a 100-fold serial dilution of RNA and the quantity of  $\beta$ actin was determined. The cDNA synthesis was replicated using identical conditions. In each case there was not a linear relationship between the initial RNA and cDNA yield. (B) A constant copy number ( $10^4$ ) of a specific sequence is added to the 100-fold RNA dilution and the specific target detected by qPCR. A higher concentration of spike sequence molecules was detected in the sample containing the lowest concentration of background RNA and a lower concentration of spike molecules was detected in the samples containing a higher concentration of RNA. The same number of copies of the spiked sequence was detected in samples containing both 100 pg and 1 ng.

using qPCR. Since exactly the same number of molecules was added to each RNA sample, the same Ct should be produced. In contrast, more spike sequence molecules were detected in the sample containing the lowest concentration of background RNA and a lower concentration of spike molecules was detected in the samples containing a higher concentration of RNA. The same number of copies of the spiked sequence was detected in samples containing both 100 pg and 1 ng. As observed for the RT reaction, there was a non-linear inverse relationship between the number of spike targets detected and the background concentration of cDNA (Figure 9.6B).

A similar phenomenon was also reported by Stahlberg *et al.*<sup>33</sup> who also demonstrated that this effect can be relieved by the addition of carriers such as PEG.

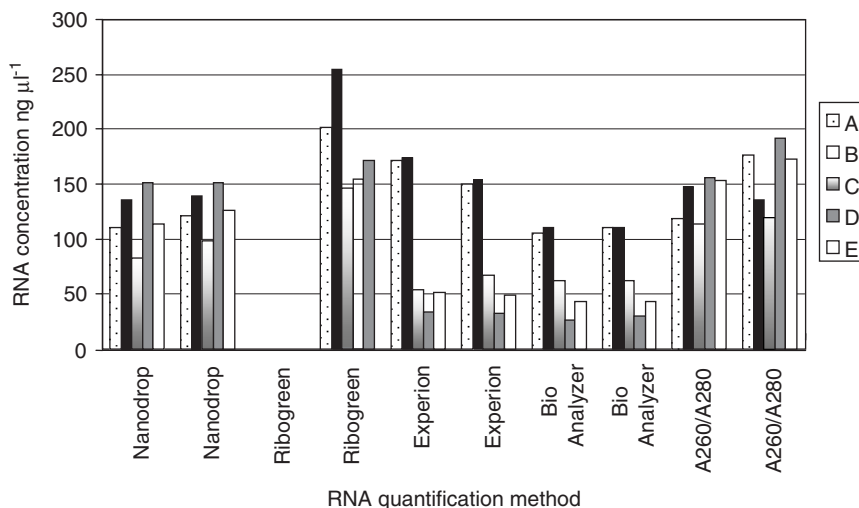
## 9.4.2 Methods of Quantification

It is for these reasons that RNA samples should be quantified after extraction whenever possible. In the absence of a perfect nucleic acid quantification system, the approach which is most suitable for the laboratory should be used. The NanoDrop<sup>®</sup> system (NanoDrop Technologies, USA) has a wide, dynamic range of quantification but is labour intensive because it only processes a single sample at a time; the chip analysis systems from Bio-Rad and Agilent process up to twelve samples simultaneously and provide measures of RNA quality (but see Section 9.3.1 and below for an assessment of these measurements). The disadvantage of these systems is that they are relatively expensive. When more samples are to be quantified, RiboGreen<sup>®</sup> staining (Molecular Probes, Invitrogen, USA) is a practical approach. This is a simple binding dye assay and can be carried out using a fluorescence plate reader or any qPCR system that has an integral sample fluorescence read function.

It has been demonstrated that when exactly the same samples are quantified using different quantification methods the results are wildly different.<sup>34</sup> An example of a comparison between quantification values is shown in Figure 9.7. There are similarities between the NanoDrop<sup>®</sup> and spectrophotometric quantities because these both use  $A_{260}$  conversions. The Agilent Bioanalyzer and Bio-Rad Experion are also similar, although the absolute values derived from the Experion are consistently lower than those from the Bioanalyzer. The RiboGreen<sup>®</sup> determination of RNA quantities was generally higher than that of any other system. It is striking that the different approaches resulted in a different quantification and that the relationship between these values is not consistent. Whichever quantification system is selected it is critically important to use the same system for all samples which are to be included in a given experiment.

## 9.5 Effect of RT Experimental Design on qPCR Data

It has been demonstrated that it is necessary to correct for the lack of linearity between the concentration of RNA and the cDNA yield. This can be achieved by addition of carrier<sup>35</sup> or inclusion of exactly the same concentration of RNA



**Figure 9.7** Comparability of RNA quantification methods. RNA samples A and B consisted of purified RNA samples, normalised to  $100 \text{ ng } \mu\text{l}^{-1}$  with reference to Nanodrop<sup>®</sup> quantification, C was prepared by degradation of sample A, samples D and E were prepared by addition of EDTA to sample A to final concentrations of 125 mM and 62.5 mM respectively. The concentration of each sample was determined from independent duplicate measurements using the Agilent Bioanalyzer, Bio-Rad Experion, NanoDrop<sup>®</sup>, RiboGreen<sup>®</sup> and conventional spectrophotometry reading at  $A_{260}$ . The duplicate readings using identical systems are highly reproducible. (Data kindly provided by students attending the qPCR training course at EMBL, Heidelberg.)

into the qRT-PCR reaction. The latter is the most usual and it is reasonable to expect that replicated samples would produce exactly the same cDNA profile. Many experimental designs rely upon periodic acquisition of clinical samples that are processed in batches. The data from a typical experiment are shown in Figure 9.8. Total RNA was extracted from clinical samples, quantified and then global cDNA produced using random nonamer primers. Each batch of clinical samples was processed alongside a positive control RNA sample (human reference RNA; Stratagene). The cDNA samples were then interrogated for the quantities of the transcripts of interest with reference to a serial dilution of human reference RNA (Stratagene). Transcript quantities of the test gene in the cDNA of two batches of clinical samples and both positive controls are shown in Figure 9.8A and the quantification of  $\beta$ actin in the same samples is shown in Figure 9.8B. Analysis of the test gene transcript quantities indicates that this is present at lower levels in the first set of samples than in the second set. The comparison of the quantities in the controls (calibrator reference samples) associated with the two batches reveals that the reverse transcription was less efficient for the first calibrator reference sample. The most common procedure to correct for differences in reverse transcription efficiency is to refer the gene of

interest to one or more stable reference genes. This technique is performed with the expectation that the reverse transcription of all transcripts is equally efficient in all samples. Analysis of the quantity of  $\beta$ actin in the same cDNA samples reveals that this is not a safe assumption. In contrast to the test gene profile, a higher yield of  $\beta$ actin is detected in the first batch of samples and this is also reflected in the calibrator reference samples. This phenomenon was explored further by comparison of the quantity of three genes in the identical calibrator reference samples processed on four independent occasions. Despite all practical variables being constant, the relative quantity of different transcripts varied between the apparently identical reverse transcription reactions (Figure 9.8). It is evident from these data that reverse transcription reactions are not always reproducible between batches and that the variability does not maintain the proportionality of transcript quantity.<sup>36–37</sup>

An alternative method for construction of a calibration curve is to dilute total RNA and detect the specific target using target-specific priming. Under these experimental conditions, in contrast to the use of random priming, there

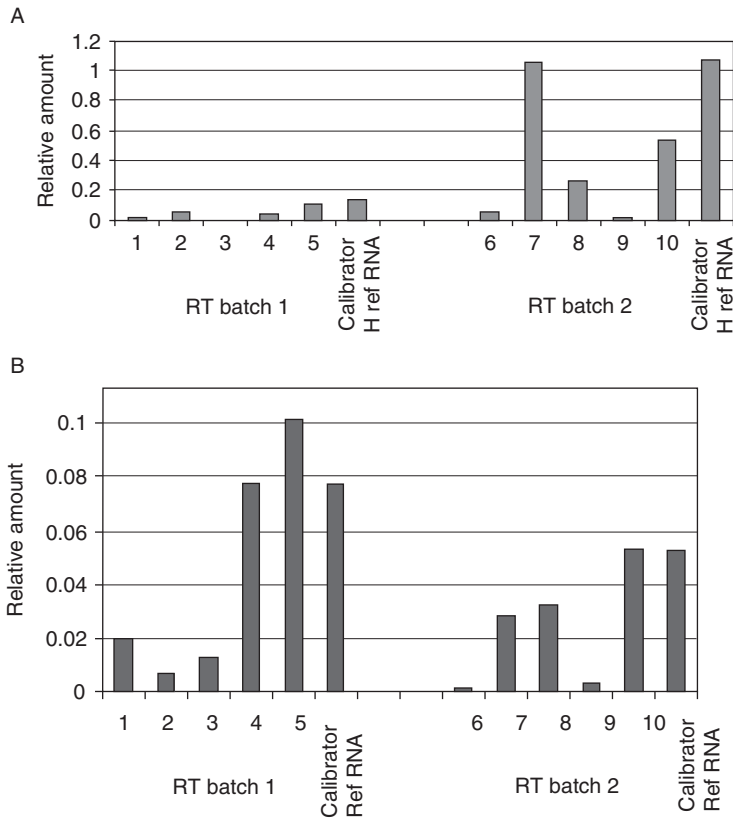
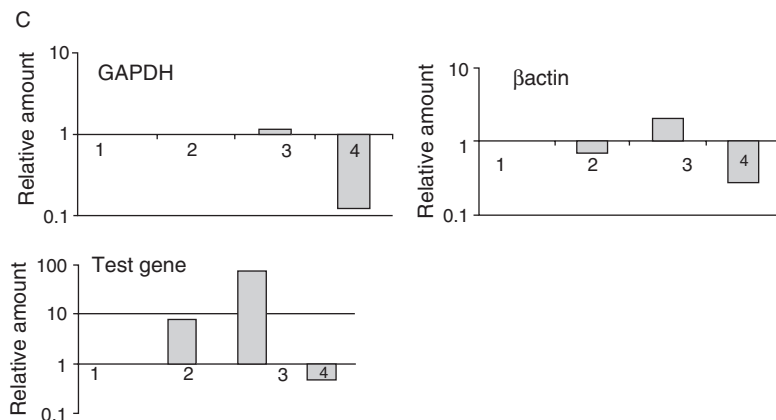


Figure 9.8 (Continued)



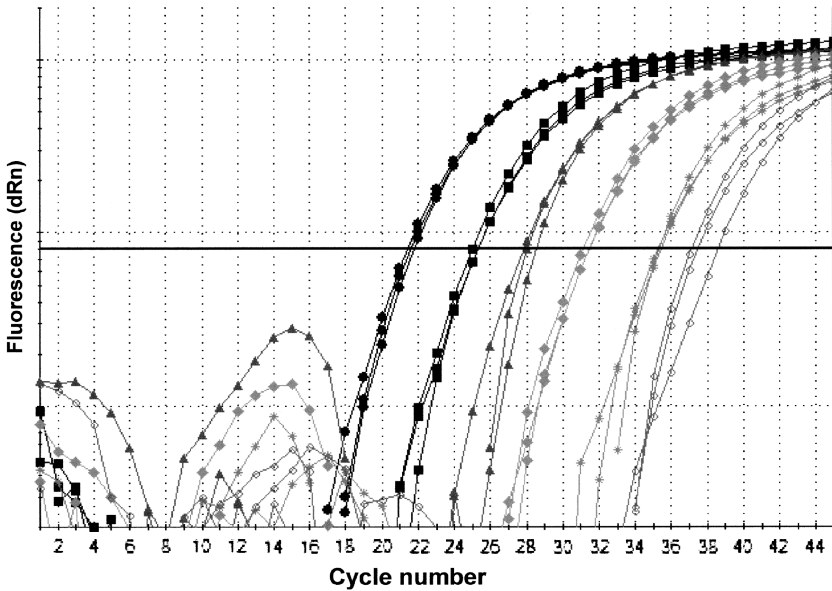
**Figure 9.8** The quantity of each specific gene target was determined in each cDNA sample and is expressed relative to the quantity detected in the universal standard curve (constructed from a serial dilution of cDNA). (A) The transcript quantities of the test gene in the cDNA clinical samples and positive controls were different between two batches of RT reactions such that the test gene transcript quantities are lower in the first set of samples than in the second set. (B) The quantification of  $\beta$ actin in the same samples indicates that a higher yield of  $\beta$ actin is detected in the first batch of samples and this is also reflected in the calibrator reference samples. (C) The quantity of three genes, GAPDH,  $\beta$ actin and test gene, were determined in identical calibrator reference samples processed on four independent occasions. The graphs show the data from each quantification relative to the quantity of the target recorded in the first experiment. There were similar quantities of GAPDH in the first three replicated cDNA samples with a relative decrease of around eight-fold in the 4th sample. There was more variability in the  $\beta$ actin quantities in samples 2 and 3, with only a six-fold decrease recorded in the 4th sample. There was much more variability in the quantities recorded for the test gene with a 72-fold increase in the 3rd sample and a three-fold decrease recorded in the 4th sample. The RT reaction was variable between batches and transcript proportionality was not maintained.

is a linear relationship between the initial concentration of RNA and the yield of specific target (Figure 9.9).

## 9.6 Conclusion

The qRT-PCR is undoubtedly the method of choice for quantification of specific RNA targets. However, in order to produce reliable mRNA quantification data it is critical to ensure that each stage of the process is optimal; all processes require validation including RNA extraction and quantification, template quality assurance assessment, reverse transcription reproducibility and finally qPCR assay optimisation. Until each of these processes is standardised and the information to demonstrate that these procedures have been carefully





**Figure 9.9** A calibration curve constructed by dilution of total RNA and GAPDH transcript detected using target specific priming. In this case there is a linear relationship between the initial concentration of RNA and the yield of the specific target.

controlled is included in peer-reviewed papers it will remain almost impossible to compare the wide range of reports due to lack of technical compatibility. Worse still, lack of control over any one of the required procedures can lead to meaningless numbers gaining apparent validity due to statistical analysis. Simply, validation matters.

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