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qPCR

RNA quality matters

Mikael Kubista, Jens Björkman, David Svec and Robert Sjöback
TATAA Biocenter

RNA levels can be measured with very high specificity, sensitivity and accuracy with techniques such as real-time quantitative PCR (qPCR), microarray analysis and next generation sequencing. This makes messenger (m) RNAs and potentially microRNAs and other non-coding RNAs popular as biomarkers. But RNA is less stable and more dynamic than DNA, and assays are not always specific for RNA, so can we trust measured expression values?

A biomarker is a biological molecule found in blood, other body fluids or tissues, and is a sign of a normal or abnormal process, or of a condition or disease[1]. The biomarker may be used to see how well the body responds to a treatment for a disease or condition. Most popular and common molecular biomarkers are DNA, RNA and proteins. While proteins and in particular DNA are quite stable molecules and can be analysed for many properties such as sequence years after being removed from their natural biological environment, RNA molecules are not (**Table 1**). The extra 2'-hydroxyl group on the ribose in RNA that is absent in DNA is a nucleophile. It confers catalytic activity to ribozymes, but also makes RNA intrinsically unstable. In aqueous solution, RNA spontaneously degrades through self-cleavage catalysed by metal ions such as Mg^{2+}, high (>9) or low (<2) pH, and temperature. EDTA or citrate is therefore typically added to RNA preserving solutions to chelate Mg^{2+}[2]. Although RNA is more resistant to ultraviolet (UV) irradiation than DNA, it causes several types of damage including photochemical modification, cross-linking and oxidation. RNA solutions should therefore be stored in dark or in vessels with lids that do not transmit UV light. Spectroscopic measurements with UV light may damage the RNA (and DNA), particularly under extensive illumination. In those cases, damage can be reduced by degassing the solution removing oxygen. RNA is also prone to degradation by RNases.

Table 1 Confounding effects influencing measured RNA levels

Phenomenon	Affected by
Self-cleavage	Mg^{2+}, pH, temperature
Chemical modification/degradation	Aldehydes (formalin), Paraffin
Physical degradation	UV, oxygen
Nucleolytic degradation	Exo and endonucleases
Induction (repression) of transcription	Change of environment
Adsorption	Surface adsorption
Extraction	Physical losses
Reverse transcription	Priming, enzyme, target dependence
PCR, hybridisation	Target dependence

There are many types of RNases including 3' and 5' exonucleases as well as endonucleases. RNases are incredibly stable enzymes that withstand heat as they rapidly refold upon cooling; they are not even fully destroyed by autoclaving. Chaotropic salts such as guanidinium will temporarily inhibit RNases, but irreversible inhibition requires treatment with beta mercaptoethanol or DEPC (diethylpyrocarbonate), which reacts with the catalytic amino acid in the reactive site of the RNase molecule. Excess DEPC can then be inactivated by incubation at 37°C. It is also possible to inhibit RNases with RNase inhibitors. RNases are highly abundant and doing RNA work precautions should be taken such as using RNase free tips, tubes and solutions (including water), and using gloves that are changed regularly. Benchtop, pipettors and glassware used shall be decontaminated for RNA work. Interestingly, while RNases are a major complication when working with classical bulk samples, it is usually not a problem in single cell expression profiling[3], because most RNases are secreted and removed by careful washing of the cell before lysis. For the same reason, RNA in samples that have been freeze-thawed, which bursts the cells, are much more prone to degradation than sample with intact cells. Tissue specimens preserved for molecular analysis are

often formalin-fixed and paraffin-embedded (FFPE). This procedure severely damages the RNA by modifying its basis and degrading the strands[4]. Details of the protocol used and also the length and conditions of storage have a profound effect of the RNA quality, which is often very poor with detrimental consequences on the precision of downstream mRNA measurements.

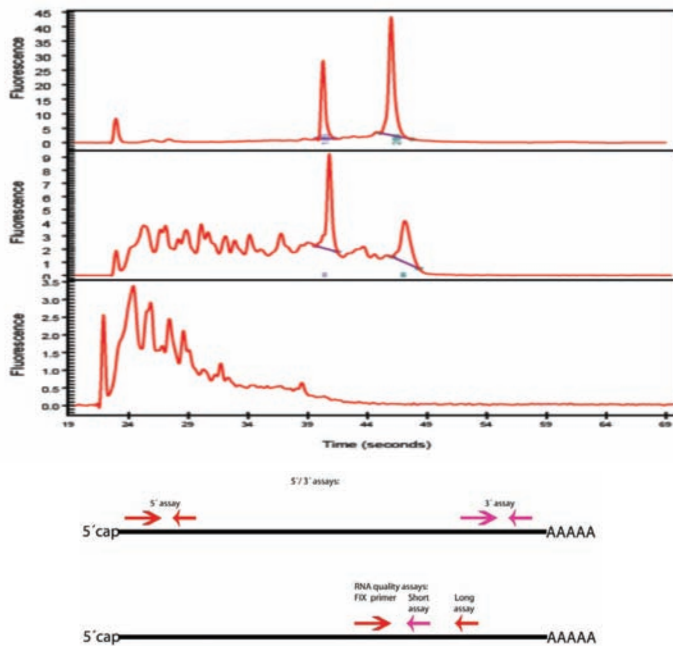

Figure 1A Assessing RNA integrity. Top: electropherogram (top panel: intact RNA with RIN 10, middle panel: partially degraded RNA with RIN 5; bottom panel: heavily degraded RA with RIN 3). Middle: 3'/5' strategy to assess mRNA degradation; Bottom: long/short strategy to assess mRNA integrity

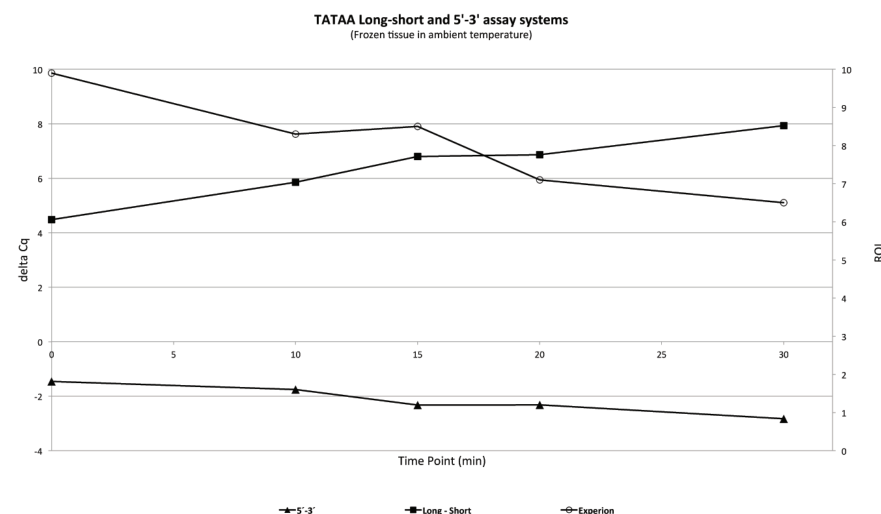

Figure 1B Assessing RNA integrity. Comparison of RQI from electropherograms measured with the BioRad Experion with 3'/5' assay and long/short quality assay of TBP on mouse tissue degraded in room temperature

There are some strategies to reduce the effect of poor RNA quality on RT-qPCR analyses, including designing assays that produce short amplicons that are of equal size for the genes that shall be compared[5]. But the rule is, poor quality in, poor quality out. If RNA quality is poor there is little to do. It is therefore desirable to learn quality is poor before spending resources on analyses that will not be reliable. Novel methods for preservation of biological specimens that preserve morphology as well as RNA/DNA integrity have been developed[6], but it will take time before these become routinely used, since most archival material that new samples are compared to is FFPE. The most common method to test RNA quality is by analysing the sample using electrophoresis separating the RNA present by length and analysing the length distribution. The strategy relies on ribosomal RNA, which is the dominant species, and in intact samples, two bands reflecting 18S and 28S RNA are expected. Most often, a microfluidic device is used, such as the Bioanalyzer[7], Experion[8] and the LabChip[9], the more recent TapeStation[10] or capillary electrophoresis in the QIAxel[11] or the Fragment Analyzer[12]. The instruments generate an electropherogram, which is a digital representation of the electrophoretic pattern showing the appearance of fragments over time and can be analysed to calculate an RNA quality score. Best known is the RIN (RNA integrity number) obtained from the Bioanalyzer, which is a number between 1 and 10 calculated by identifying features in the electropherogram and assessing those using an algorithm developed based on a large training set of electropherograms recorded on RNAs of various origins and different degree of degradation[13]. An RIN of 10 reflects intact RNA and the lower the RIN, the more degraded the RNA is (**Figure 1**). Other instruments provide similar quality indexes, but because of independent learning algorithms, they are not really convertible. While the RIN or equivalent indexes sensitively reflect minor degradation of RNA, they are less robust for the assessment of highly degraded samples. One reason is that the degradation is not particularly reproducible and the analysed features in the electropherogram become much smaller and less distinct. Furthermore, as mentioned already, the electropherogram reflects the integrity of the dominant ribosomal RNA species that are chemically and structurally different from the mRNAs.

Ribosomal RNAs lack cap as well as A-tail and are folded into tight three dimensional structures, resulting in quite different molecular stability and sensitivity to various degrading agents from mRNAs. Hence, even if we could assess extensive degradation of ribosomal RNAs with high precision using capillary electrophoresis, it would still not reflect the integrity of the relevant mRNA species. Measurements of RIN or equivalent indexes should therefore be taken as a general indicator of sample quality, which is valuable, for example, to compare sampling, transport and storage conditions, rather than assessment of mRNA. mRNA quality for expression analysis is better assessed using molecular methods. The first method described was the 3'/5' assay (**Figure 1a and b**)[14]. The mRNA is supposed to be exclusively reverse transcribed from the 3'-end using

oligo(T) primer and the amount of transcript produced is measured by qPCR targeting a sequence close to the mRNA 3′-end and another sequence close to the mRNA 5′-end. Since reverse transcription is initiated at the mRNA 3′-end, amplification of the sequence at its 5′-end will be successful only if the mRNA was intact. Hence, comparing the amounts of the two amplicons produced should reflect the integrity of the targeted mRNA. The idea is excellent and the approach works well under some conditions, but we have found it fails to perform under many conditions where heavily degraded samples are analysed. Presumably, these samples contain fragments of DNA that can act as primers and, since RT is not particularly sensitive to the priming event[15], the required exclusive priming at the 3′-end is not achieved.

A more recent approach is the short / long quality assay (**Figure 1a and b**, page 64)[16]. The cDNA is amplified producing two amplicons of different length using one common primer. If the mRNA was fragmented, higher yield is expected of the shorter amplicon. The cDNA can be produced with any priming strategy and the approach is therefore not sensitive to the presence of fragmented DNA in the sample.

When biological samples are collected, the material is withdrawn from its natural environment and placed in a highly artificial surrounding. Unless the cells are immediately fixed, lysed or otherwise killed they will respond to the altered conditions. The response can be dramatic, with some genes being up or down regulated manifold and the changes can go on for days[17]. When collecting blood for gene expression analysis, for example, it is critical to immediately preserve the profiles using special collection tubes such as the PAXgene[18] rather than in EDTA, which perturbs the expression. Recently, the European project SPIDIA[19] performed proficiency ring trial to assess the variation in performance between European routine laboratories and to verify the influence of EDTA on measured mRNA levels[20].

Although some technologies such as the northern blot, mRNA microarrays and Nanostring analyse mRNAs directly, the gold standard qPCR and next generation sequencing techniques require mRNA is converted to cDNA for analysis. This is done by reverse transcription, which is a reaction employing primers and reverse transcriptase. Three different priming strategies are used: gene specific primers, random sequence primers and oligothymidine primers, and the reverse transcriptase is typically an engineered variant of either Avian Myeloblastosis Virus (AMV) or Moloney Murine Leukemia Virus (MMLV). The reaction can be performed over a range of conditions including different temperatures[15]. All these factors influence the reverse transcription yield, which varies up to 200-fold depending on the priming strategy, enzyme, reaction condition and target[21]. The reverse transcription is also prone to inhibition. Following reverse transcription, qPCR is generally highly efficient reaction and qualified assay suppliers typically guarantee PCR efficiencies of at least 80 per cent in the absence of interference. However, those that compare PCR efficiency of a purified template with that of real samples with template in complex matrixes experience inhibition[22]. For the same amount of template, Cq's in pure media are lower than Cq's in complex matrices due to interference with reaction components. Thus, the presence of substances that

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interfere with amplification will directly influence the performance of PCR and may also limit sensitivity.

For analysis of complex samples, it is therefore recommended to determine the sensitivity of the assay, preferably expressed as the limit of detection[23]. Some inhibitors dramatically interfere with amplification, even at very small amounts. For example, PCR mixtures based on the widely used Taq DNA polymerase are totally inhibited in the presence of 0.004 per cent (v/v) human blood[24]. The PCR inhibitors originate either from the original sample or from sample preparation prior to PCR or from both[25,26]. Examples of inhibiting substances present in original samples include bile salts and complex polysaccharides in faeces[27,28], collagen in food samples[29], heme[24], immunoglobulin G (IgG)[30], and lactoferrin in blood[31], humic substances in soil[32,33], melanin and myoglobin in tissue[34-36], polysaccharides in plants[37], proteinases and calcium ions in milk[38], indigo dye in denim[39], and urea in urine[40]. Fatty tissues are in general very problematic. Components from sampling and extraction that inhibit PCR include chelators such as EDTA, which complexes Mg^{2+}. Interestingly, trace amounts of phenol inhibit Taq polymerase, while Tth polymerase maintains both DNA and RNA-dependent DNA polymerase activity in the presence of five per cent (v/v) phenol. Excess of KCl, NaCl and other salts, ionic detergents such as sodium deoxycholate, sarkosyl and sodium dodecyl sulphate (SDS) also inhibit PCR[41], as well as alcohols such as ethanol and isopropanol[42]. Active reverse transcriptase brought over from the reverse transcription reaction can have an inhibitory effect on the PCR[43-45], and stimulate primer-dimer formation[46]. The effect is profound during the first cycles and declines as the reverse transcriptase denatures by the applied heat and as DNA accumulates. Apparent PCR efficiencies of above 100 per cent that are occasionally reported[47,48], may be obtained when standard curves are constructed based on serial dilution of cDNA, which also dilutes the contaminating reverse transcriptase[49]. To achieve the highest possible sensitivity in RT-PCR, it is pertinent to avoid the inhibiting effect of the reverse transcriptase. To test for inhibition, one can spike in internal controls. Adding a DNA spike into the PCR test sample and the same amount into a control sample, comparing the Cq values will reveal any PCR inhibition. For RNA analysis, an RNA spike can be added into the sample for reverse transcription and the same amount into a control. Comparing the Cq values will reveal issues in the RT-PCR process[50]. Adding the RNA even earlier, into e.g. the lysate or the homogenised sample, a comparison with control will reveal the performance of the entire sample pre-processing procedure. Generally, the earlier the control is spiked in the more

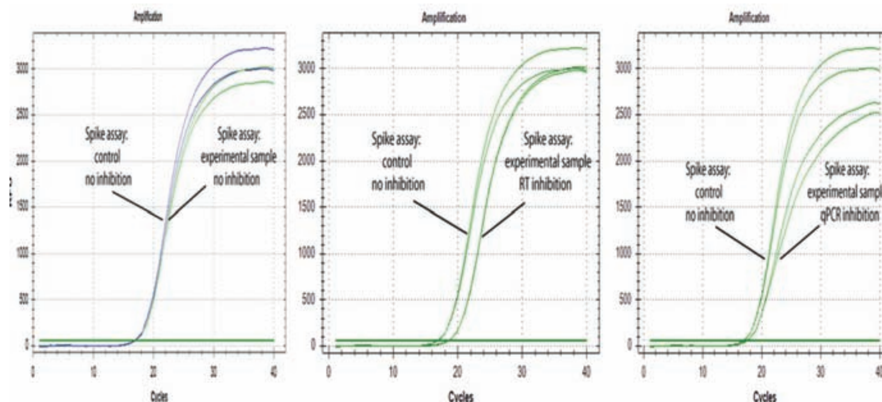

Figure 2 Test for inhibition using an RNA spike. Left: no inhibition; center: RT inhibition; right: PCR inhibition

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of the experimental procedure is validated. The ultimate control is to inject the RNA into the tissue, which then also monitors the extraction yield. To mimic the behaviour of native mRNAs, the control RNA should have a cap and A-tail[16]. Inspecting the qPCR response curves inhibition of the reverse transcription and of PCR can be distinguished. While (any) inhibition delays the response, shifting Cq to higher values PCR inhibition also reduces the slope of the qPCR response curve (**Figure 2**, page 66).

Extracted RNA is often contaminated by DNA. Since mRNAs are primary transcripts of the genes with introns removed by splicing, amplification of the genomic copy can usually be avoided by designing PCR assays with primers spanning an intron. If the intron is significant, the genomic amplicon will be too long to be amplified efficiently using normal PCR elongation time. Of course, this design strategy only works if the gene has introns. But also in those cases, intron spanning assays may produce products from genomic DNA. Eukaryotic genomes contain pseudogenes that are either gene duplications or processed mRNAs. While the former maintain the introns of the native genes, the latter are the result of random integration of reverse-transcribed mature RNA molecules and are characterised by lack of introns, and the processed pseudogenes are amplified even with intron spanning primers. Processed pseudogenes are common in mammalian species although they are much less abundant in other animals[51]. A comprehensive database of pseudogenes is available[52]. Measuring gene expression in mammals genomic DNA (gDNA) may pose a serious problem due to the presence of the processed pseudogenes. In these studies, it is pertinent to control for the gDNA background. This can be done by performing RT- controls. An RT- control is a parallel reaction where the reverse transcriptase is left out in the reverse transcription. Hence, no cDNA is formed and the RT- control assesses the background contribution from the gDNA. As a rule of thumb, the Cq value of the RT- control shall be at least five cycles above the Cq of the normal RT+ reaction, which indicates DNA contamination is less than five per cent. The approach works well, but is expensive. Recently, the ValidPrime approach was described which is more cost efficient way to assess gDNA background in RT-qPCR[53]. ValidPrime is an assay that amplifies genomic DNA only, cDNA is not amplified, and is used to measure amount of gDNA present in a cDNA preparation. In addition to testing all samples for gDNA contamination with ValidPrime, all the assays used are tested for sensitivity to gDNA by analysing a gDNA control[16]. From the combination of the two measurements, gDNA contributions to the Cq values are readily subtracted. When analysing m genes in n samples, the ValidPrime approach requires only m+n+1 control qPCR's and no additional RT, while regular RT- controls require a parallel RT followed by m*n control qPCRs. With ValidPrime using GenEx it is possible to correct for the signal from up to 50 – 60 per cent gDNA contamination[54]. Should contamination be more serious the gDNA has to be removed with DNase. Particularly convenient is to use the heat-labile double-strand specific DNase from Arcticzymes[55]. The DNase, originally extracted from shrimp, cleaves only double-stranded (genomic) DNA and will not degrade primers. It is then heat-inactivated during the PCR and will not degrade the PCR product either.

In summary, we have excellent methods to measure cDNA levels, with RT-qPCR being the golden standard. However, there are many factors that can influence the result of the measurement yielding values that do not correctly reflect the biologically relevant expression levels. A number of tools to control and test the quality of the experimental approaches are appearing and have been summarised here. The tools comply with current MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines[56]. Approaches for the design and optimisation of RT-qPCR studies have been published[57], and since recently courses on quality control and assessment of qPCR data are available[58].

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