

## REVIEW PAPER

# RNA quality assessment: a view from plant qPCR studies

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## Abstract

Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) is probably the most common molecular technique used in transcriptome analyses today. The simplicity of the technology and associated protocols that generate results without the need to understand the underlying principles has made RT-qPCR the method of choice for RNA quantification. Rather than the 'gold standard technology' often used to describe it, the performance of RT-qPCR suffers from considerable pitfalls during general workflow. The inconsistency of conventional methods for the evaluation of RNA quality and its influence on qPCR performance as well as stability of reference genes is summarized and discussed here.

**Key words:** MIQE, qPCR, 3':5' ratio, RNA integrity.

## Introduction

Since the relatively recent introduction of quantitative polymerase chain reaction (qPCR) in the plant science community, interest in the technique has increased exponentially (Gachon, 2004). While sensitive and precise, one major drawback of the technique is the numerous critical steps required throughout the entire workflow that may influence the accuracy and reliability of results (Huggett *et al.*, 2005; Derveaux *et al.*, 2010; Huggett and Bustin, 2011). The apparent simplicity of the qPCR technology has made it vulnerable to a lack of clarity and transparency in the literature, leading to few publications reporting in detail how results have been obtained. Therefore, in spite of its superiority over other methods available for evaluating gene expression, reverse transcription-qPCR (RT-qPCR) remains underused, due in part to conflicting results and difficulty in replicating experiments. While the technology itself is not intrinsically inaccurate, the absence of strict guidelines has led to researchers performing experiments and analysing data based on information gathered from disparate sources, resulting in data of variable quality (Taylor *et al.*, 2010). Two of the main obstacles impeding a more extensive adoption of RT-qPCR assays are concerns over quality assessment and data normalization, both

of which affect reproducibility. Efforts to adopt methods for the systematic validation of reference genes applying a robust normalization strategy are growing and are currently being led by molecular researchers from the medical field (Dhedra *et al.*, 2004, 2005; Radonić *et al.*, 2004; Vandesompele *et al.*, 2009). In recent years, the plant scientific community has gradually recognized the need for robust validation (Gutierrez *et al.*, 2008a, b; Udvardi *et al.*, 2008; Guenin *et al.*, 2009).

The other main issue is related to the quality of the template. This is arguably the most important determinant of the reproducibility and biological relevance of subsequent RT-qPCR results (Bustin, 2002; Bustin and Nolan, 2004; Huggett *et al.*, 2005; Imbeaud *et al.*, 2005; Pérez-Novo *et al.*, 2005; Fleige and Pfaffl, 2006; Fleige *et al.*, 2006; Pfaffl, 2010). To this end, numerous articles have elaborated on the theme of producing high-quality and reliable data from RT-qPCR highlighting the importance of RNA sample quality (Becker *et al.*, 2010; Taylor *et al.*, 2010). The instability of RNA and its sensitivity to degradation (introduced during storage under suboptimal conditions, the variety of additional steps set out in the isolation protocols, or interlaboratory sample shipments) is well known by the RNA

research community. Assessing transcript quantification confronts the essential problem of whether detected differences in gene expression are related to the hypothetical assumptions of a study or whether they are the result of a certain bias in the composition of the sample set. The ultimate goal here is that results should reflect true biological differences (data of biological significance) rather than differences due to poor RNA quality (data of statistical significance). Acknowledgement of this potential conflict should lead to a widespread debate about which operational procedure is the standard for RNA quality assessment and which requirements must be met from a technical point of view. However, current proposals for adequate RNA integrity measurements are not taken into account, and quality control in gene expression studies is often ignored. This is especially problematic in the plant field research.

Figure 1 shows the distribution of methods that have been used to perform RNA quality assessment from 520 studies based on RNA transcription analysis published in three leading high-impact journals in the past 5 years: *Journal of Experimental Botany*, *BMC Plant Biology*, and *Plant Biotechnology Journal*. The aforementioned time period also includes the seminal Minimum Information for publication of Quantitative real-time Experiments (MIQE) in April 2009 (Bustin *et al.*, 2009). Of the papers reporting RNA quality control, 1 in 2.2 addressed it using two alternative methods. Additionally, 14% of studies performed quality checks based on 15- to 20-year-old methods, though this is certainly not the best option (Fig. 1A). By far, the most difficult data to explain are the >74% of gene expression papers published that have not even mentioned performing RNA quality assessment (Fig. 1B). The low percentage of papers including RNA quality reports in the last 5 years raises the question of whether the importance of the critical sample quality control section of the MIQE guidelines has fully penetrated the plant research community. It is evident from these data that this area requires urgent attention and warrants considerable changes to the way qPCR assays are both performed and reported.

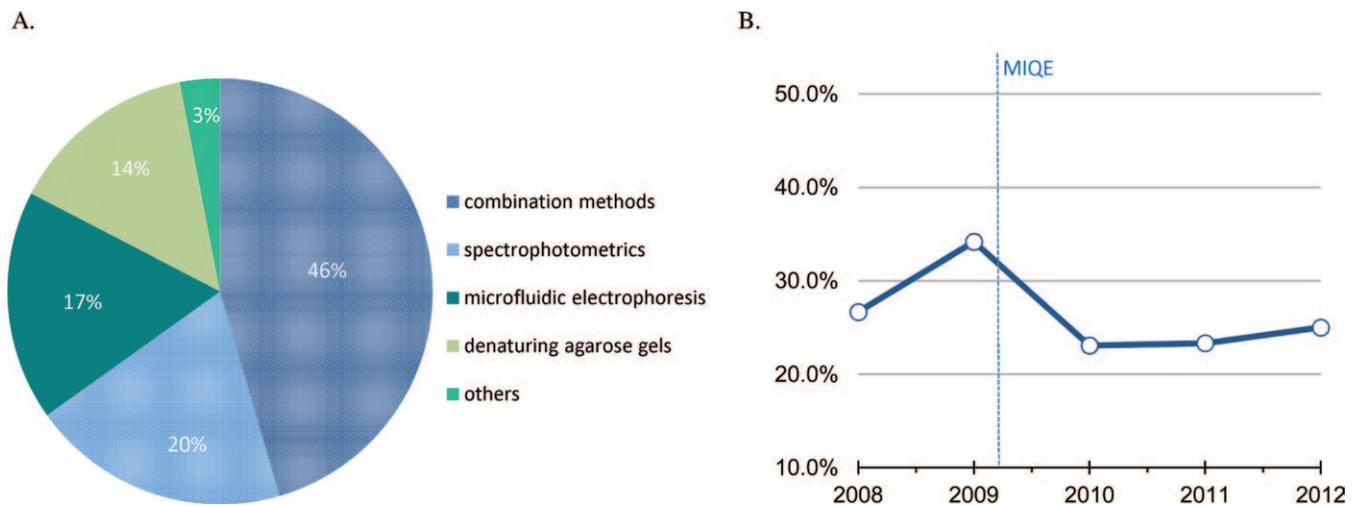
This review aims to address the effect of RNA quality on gene expression profiling using qPCR data, and to chart the progress of quality assessments in the context of high technology requiring rigid quality controls.

## Total RNA quality assessment

RNA quality has been defined as the combination of RNA purity and RNA integrity (Becker *et al.*, 2010). Purity and integrity are unrelated measures and should therefore be assessed independently, but most common techniques for controlling the quality of RNA have focused on purity. The use of techniques emphasizing purity was acceptable for conventional or end-point PCR assays, but translating such assays directly into the qPCR format is just not possible. When the sample is to be used for measurements of transcript quantity, another relevant measurement is a determination of whether the mRNA molecules are intact or fragmented, namely RNA integrity (Nolan and Bustin, 2008).

### Denaturing agarose gels

Conventional methods based on RNA electrophoretic separation aim at establishing an environment for complete denaturation in order to disrupt fully the hydrogen bonds which hamper the estimation of RNA molecular weights. The most widespread method makes use of MOPS/formaldehyde gel electrophoresis stained with ethidium bromide, though faster and safer alternatives have been developed (Masek *et al.*, 2005; Aranda *et al.*, 2012). The method relies on the assumption that rRNA quality reflects that of the underlying mRNA population. rRNA quality is assessed visually using the intensities of ribosomal bands, with a ratio of ~2 considered as a good indicator of high level integrity (Sambrook *et al.*, 1989). However as the appearance of rRNA bands is affected by the electrophoretic conditions, the amount of loaded RNA, and the saturation of ethidium bromide fluorescence, this method can be less than reliable. The main



**Fig. 1.** RNA quality assessment in gene expression studies. (a) Distribution of methods used to perform RNA quality check from studies published in three leading plant journals in the past 5 years. Data recorded until May 2012. (b) Percentage of published papers reporting to have performed RNA quality assessment from 2008 to May 2012. MIQE guidelines publication in April 2009 is shown.

disadvantage of gel electrophoresis is the significant amount of precious RNA required (typically on the order of 300–800 ng of RNA), as well as a dependency on the perception of the researcher (low-throughput method). Furthermore, the initial premise is considered questionable because it is not clear how rRNA degradation actually reflects the quality of the underlying mRNA population (Dotti and Bonin, 2011). The lack of sensitivity and specificity of agarose gels is especially clear in their inability to detect small RNA degradation (Imbeaud *et al.*, 2005; Fleige and Pfaffl, 2006). It should be further stated that this method relies on the use of formaldehyde and ethidium bromide, both toxic chemicals requiring special handling and waste disposal procedures (Sambrook *et al.*, 1989). Though RNA quality assessment methods have advanced well beyond denaturing agarose gels, confidence in RNA electrophoresis analysis has continued, due in part to reliance on traditional reasoning over data produced through more reliable methods.

### *Spectrophotometric measurement*

Another common technique for assessing the quality of RNA is optical density (OD) measurement. RNA purity can be verified on the basis of the 260/280 ratio. An  $OD_{260/280} > 1.8$  and maximization of  $OD_{260/230}$  and  $OD_{260/320}$  are usually considered acceptable indicators of good RNA quality (Sambrook *et al.*, 1989; Manchester, 1996). However, the  $OD_{260}$  can be compromised by the presence of genomic DNA, while the  $OD_{280}$  will estimate the presence of protein but provide no hint on possible residual organic contaminants (Manchester, 1995). Imbeaud *et al.* (2005) have discussed the reliability of spectrophotometrics for RNA quality measurement, stating that the limited range of detectable substances cannot reveal the state of degradation or the integrity of the sample. Moreover, conventional  $A_{260}/A_{280}$  measurement does not detect the presence of inhibitor components that are clearly detrimental to qPCR amplification in an assay-specific manner; these inhibiting agents may be co-purified components from the biological sample or reagents used during nucleic acid extraction, and are particularly relevant to formalin-fixed, paraffin-embedded (FFPE) samples. Full discussion of these concerns lay beyond the scope of the present review, though sound articles are available on the topic (Nolan *et al.*, 2006a; Nolan and Bustin, 2008).

### *Microfluidic capillary electrophoresis*

A major improvement in RNA analysis occurred with the advent of microfluidics-based electrophoresis systems (lab-on-a-chip technology). In 1999, an automated device using microfluidics technology that provided electrophoretic separation of DNA, RNA, and protein samples, the 2100 Bioanalyzer system, was introduced (Mueller *et al.*, 2000). The first software for the instrument calculated the ratio of the two ribosomal bands, following the traditionally used approach for RNA quality assessment; this proved to be far superior to the gel-based approach, as it was free of the subjective visual interpretation. However, these ribosomal ratios showed a practical value as long as there were no prominent degradation products (Auer *et al.*, 2003; Schoor *et al.*, 2003). Moreover, when ratios were calculated from identical

samples but through independent runs, a large degree of variability was observed (Imbeaud *et al.*, 2005). Still newer technologies continue to report that ribosomal ratios show a weak correlation with RNA integrity (Pfaffl *et al.*, 2008) as well as no significant correlation between the 28S/18S ratio and qPCR performance (Fleige *et al.*, 2006).

Due to the limited reproducibility of rRNA ratios to assess RNA integrity, a user-independent classifier algorithm, the so-called RIN (RNA Integrity Number) for standardization of RNA quality control was introduced in the Bionalyzer instrument (Schroeder *et al.*, 2006). A total of 1300 electropherograms of RNA samples from various tissues for three mammalian species (human, mouse, and rat), showing varying levels of degradation, were used. An adaptive learning approach was developed in order to assign weights to the relevant features that describe the RNA integrity from the electrophoretic trace: the total RNA ratio (ratio of the area of ribosomal bands to the total area of the electropherogram), the height of the 28S peak, the fast area ratio (ratio of the area in the fast region to the total area of the electropherogram), and the height of the lower marker. The RIN was computed for each RNA profile, resulting in the classification of RNA samples in 10 numerically pre-defined categories of integrity. Thus, the output RIN is a number in the range of 1–10 (completely degraded RNA samples–intact RNA samples). Similarly, the RQI (RNA Quality Indicator) algorithm was introduced in the Experion system as a method to standardize and quantify RNA integrity (Denisov *et al.*, 2008). Here, only three regions of the electropherogram are taken into account when mapping a sample for RQI calculation: the 28S region, the 18S region, and the pre-18S regions. Comparability and validity of results in terms of RNA quality delivered by both lab-on-chip-systems have been investigated, and, overall, both algorithms have been shown to be functionally equivalent in reliably determining RNA integrity (Denisov *et al.*, 2008; Riedmaier *et al.*, 2011).

Both algorithms were first established and tested using various mammalian tissues and have since been expanded to RNA samples from a variety of organs and organisms, including bacterial RNA (Jahn *et al.*, 2008; Pinto *et al.*, 2012). Evidently, ‘lab-on-a-chip’ technology has also been widely adopted by the plant community. For example, a freely accessible database showing species where RIN has been used (though without updates) can be searched (<http://www.chem.agilent.com/RIN/>). While plants have the ubiquitous major ribosomal subunits, additional rRNA species, like those found in photosynthetic tissues, can be present as well. Electropherograms should therefore be visually inspected to confirm that ribosomal peaks have been properly identified by the software. Following these precautionary measures, RNA quality assessment with Bioanalyzer or Experion systems has been performed for a number of different topics including gene expression analysis during plant–pathogen interactions (Klink *et al.*, 2007), plant–symbiotic interactions (Grunwald *et al.*, 2009; Branscheid *et al.*, 2010), comparison between relative quantification approaches (Regier and Frey, 2010), or identification of stable reference genes (Klie and Debener, 2011; Lilly *et al.*, 2011).

The significant advantage of microfluidic systems is the significant decrease in the amount of RNA needed to evaluate integrity down to the submicrogram scale; a property which will probably

lead to its increased use in the future. Though the cost of automated electrophoresis stations renders the method unfeasible for laboratories with resource constraints, the services provided by external genomics companies are making the approach increasingly accessible.

#### The 3':5' ratio mRNA integrity assay

In the absence of an alternative, the use of a 3':5' assay has been proposed for assessing mRNA integrity (Nolan *et al.*, 2006b). The data obtained are independent of rRNA integrity, provide a reasonable measure of the degradation of the transcripts, and are modelled using the standard approach adopted by microarray users and conventional techniques applied to end-point PCR assays (Auer *et al.*, 2003). The assay is particularly applicable for analysis of precious samples when little RNA is available (Nolan and Bustin, 2008). Moreover, RNA integrity is inferred using the same technology used for RT-qPCR, giving the 3':5' assay greater relevance compared with other methods. The 3':5' ratio aims at measuring the integrity of a reference gene mRNA that is considered to be representative of the integrity of all mRNAs in a given RNA sample by amplifying different amplicons. It is based on the evidence that cDNA yield from sequence near the 5' end of partially degraded mRNAs is significantly less than from sequence near the poly(A) tail (Swift *et al.*, 2000); that is, poor RNA quality adversely affects synthesis of first-strand cDNA, resulting in under-representation of the 5' moiety of the transcript. The main advantage of using a 3':5' ratio to assess the RNA integrity is that this method specifically focuses on the integrity of an mRNA molecule instead of addressing the rRNA transcripts. As such, the 3':5' assay appears to constitute the most useful parameter to qualify RNA samples (Vermeulen *et al.*, 2011).

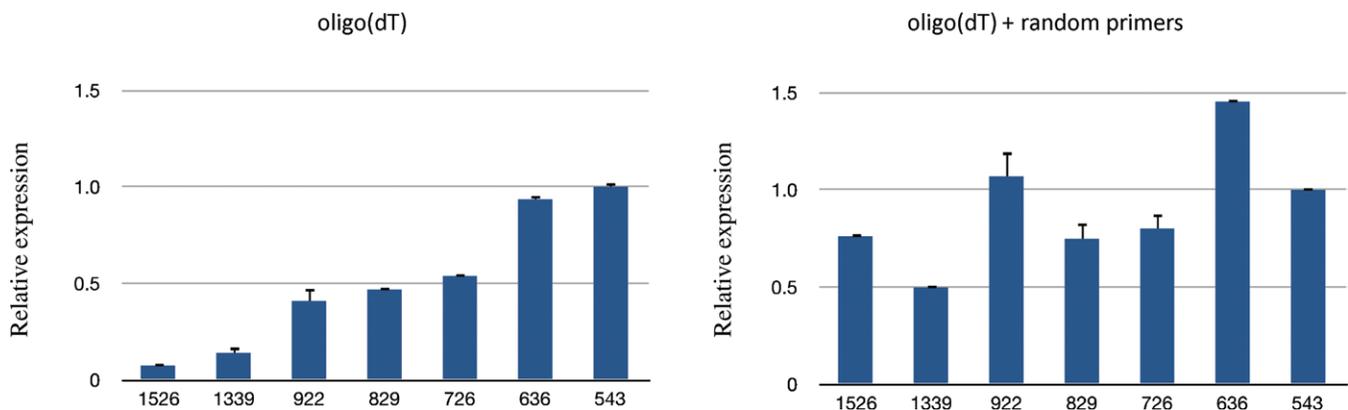
The usefulness of the 3':5' ratio relies on the oligo(dT) priming method for cDNA synthesis; consequently, the progress of the reverse transcriptase is wholly reliant on the intactness of the mRNA. Under ideal conditions, the reverse transcription will generate full-length cDNA including the 5' end of the RNA,

but the process will be interrupted wherever the mRNA is fragmented. Unlike other methods, the use of oligo(dT) and random primers is unsuited for this assay, as random sequence primers will copy RNA at multiple origins along the template and thereby produce more than one cDNA target per original mRNA target, including cDNA produced from rRNA: performed under these conditions, the final PCR yield may be somewhat higher while the template becomes partially degraded (Fig. 2).

The ideal 3':5' ratio of 1 corresponds to the highest quality material, while other ratios depend on the differing number of nucleotides between the 5' and 3' amplicons that can discriminate between different integrity levels of the RNA samples. Each amplicon may represent a 3' or 5' assay as that designation is merely of location: the 3' target assay is designed to amplify an amplicon near the 3' end, and the 5' target assay targets the 5' end of the mRNA sequence. Anchoring the 5' assay ~1500 nucleotides from the 3' end of the sequence and separating the two assays by ~1100 nucleotides characterizes a 4.43-fold difference cut-off as unreliable for downstream quantification studies (Die *et al.*, 2011).

#### Influence of RNA quality on gene expression profiling using RT-qPCR

The need for high-quality RNA standards has been an unparalleled challenge in the microarray field. Probably the cost of such technology forced researchers to recognize the influence of the issue on successful experiments. Thus, the inclusion of samples with degraded RNA has shown a significant influence on the statistical analysis and hence the interpretation of gene expression levels, leading to the conclusion that degraded samples should not be reasonably considered for analysis when using microarray technology (Schoor *et al.*, 2003; Copois *et al.*, 2007; Strand *et al.*, 2007). However nice-shaped sigmoidal amplification curves can usually be obtained with qPCR, even from degraded templates. Despite the vast number of publications pointing out that starting with low-quality RNA may strongly compromise the results of



**Fig. 2.** Normalized relative expression levels of different amplicons throughout the ubiquitin cDNA sequence (DFCI Medicago Gene Index, TC112803) from *Medicago truncatula* RNA root samples. Two different priming strategies were performed using the ImProm-II Reverse Transcription System (Promega). Bars represent mean expression level  $\pm$ SEM from two independent partially RNA degraded samples (RQI < 5). The x-axis represents amplicon distance in nucleotides from the 3' end. Normalized values are rescaled to the 543 assay.

downstream applications, quality control prior to qPCR measurement is still an often overlooked consideration (Fig. 1).

#### *Influence of RNA quality on RT-qPCR performance*

Today, it is acknowledged that RT-qPCR performance is affected by RNA integrity. Imbeaud and colleagues (2005) have shown that artefactual detection of false-positive and false-negative differential expression may be observed due to RNA integrity differences: their results indicated that up to 7-fold differences may be expected in the relative expression levels measured in samples that differ only by their quality. On the other hand, Fleige and colleagues (2006) proved a significant negative relationship between RNA quality and Cq for all samples they tested (by using RIN as quality metric). Similarly, Koppelkamm *et al.* (2011) have recently reported Cq shifts between the highest and lowest RIN values showing statistically significant correlation coefficients.

Vermeulen *et al.* (2011) have reported the most complete framework to measure the impact of RNA quality on the gene expression results to date. They studied the impact of RNA quality on the significance of differential expression of marker genes between two risk groups of cancer patients using six RNA quality parameters. Their results clearly showed an influence of RNA quality on single gene differential expression for a substantial number of genes. While all quality measures were correlated, assessments based on the mRNA rather than rRNA were the best indicators of reliable amplification.

Joining these technical studies, there are a number of other publications showing the biological relevance of high-quality RNA for obtaining reliable data from qPCR experiments. For example, Lipska and colleagues (2006), focusing on schizophrenia analyses, showed that differences in RNA quality led to crucial effects much more pronounced than underlying disease-related effects. At that time, Kerman *et al.* (2006) found significant differences in the quantification of gene expression in microdissected tissues for laser capture microdissection, showing the impact of RNA quality on the outcome of RT-qPCR studies. More recently, Taylor (2011) categorized breast cancer RNA samples by integrity based on RQI and used the minichromosome maintenance protein MCM7 as a model target gene to determine the importance of appropriate sample quality for the results. The relative expression of *mcm7* was assessed between normal samples of low quality and tumour samples, showing no significant differences, and contrasting with the opposite results when comparing normal samples of high quality and tumour samples.

#### *Influence of RNA quality on reference gene expression stability*

One of the more relevant debates is related to the elucidation of whether a data normalization step can eliminate the influence of impaired RNA integrity. The gold standard for normalization of qPCR expression data is normalization against multiple, validated reference genes (Derveaux *et al.*, 2010). The reference gene-based normalization corrects for variable starting amounts of RNA and for differences in reverse transcription efficiency, as the references are exposed to the same preparation steps as

the gene of interest (Radonić *et al.*, 2004; Bustin, 2005; Huggett *et al.*, 2005; Udvardi *et al.*, 2008). Nevertheless, it is a general misconception that the influence of degraded RNA will be corrected by the normalization step using expression of the reference genes from the same degraded RNA template. Perez-Novo and colleagues (2005) have shown that this faulty thinking may lead to misinterpretation of target gene expression level information where there is no prior knowledge of the RNA degradation status. Quantifying 10 commonly used reference genes in both intact and degraded RNA, they showed that the stability of those references was different within the same tissue types according to the degradation status of the samples. By determining the average gene-specific variation of all reference genes, the authors found that the levels of those genes were always higher in degraded versus intact samples, and therefore proposed performing RNA quality control and discarding degraded samples. Although normalization may improve mRNA quantification, even a significant difference (up to 4-fold, meaning ~75% mRNA is degraded) in gene expression may be expected in samples differing only in their quality (Fleige *et al.*, 2006); a fact which is especially relevant in accurately quantifying small differences in expression. More recent studies have confirmed the influence of RNA quality on reference gene expression stability, indicating that the process of normalization does not completely resolve the effect of compromised RNA quality on the final results (Vermeulen *et al.*, 2011).

Obviously, the greatest benefit of quality prediction prior to qPCR assays is the determination of a cut-off point from which one can move forward with additional experiments: knowing the degree to which results may be compromised may prevent substantial cost in the form of wasted reagents and technical time. Data obtained with the most degraded samples cannot be reasonably considered for downstream application, creating debate over what could be an acceptable level of degradation.

### **Gene expression profiles from partially degraded RNA**

Intact RNA obviously constitutes the best representation of the natural state of the transcriptome; however, there are situations in which gene expression analysis may be desirable even on partially degraded RNA. In plant research, as in other disciplines, there is an increasing interest in extracting nucleic acids from FFPE samples. Nevertheless, a major challenge of FFPE material is related to the extensive degradation of RNA due to the fixation procedure. Given the fact that patient samples in human clinical studies are extremely valuable, it is not surprising that most of the attempts to address the utility of partially degraded RNA from non-ideal samples came from the biomedical research field. To our knowledge, Schoor and colleagues (2003) were the first to study the quality of RNA preparations in the context of gene expression analysis by microarrays. These investigators were interested in the impact of varying amounts of RNA degradation on the expression profile of the samples, inducing RNA degradation in human tumour and healthy tissue samples by endogenous RNases. The study established that expression differences from partially degraded RNA samples with still

visible ribosomal bands were similar to those obtained from high-quality samples. Some moderate degradation therefore does not preclude microarray analyses and it might still lead to meaningful results if used carefully. In the context of qPCR, the points that deserve attention are as follows.

- (i) The RT-qPCR technique could be particularly suitable for quantifying mRNA levels in tissue samples containing partially degraded RNA due to the short length of amplicons (Bustin, 2002; Antonov *et al.*, 2005; Fleige *et al.*, 2006; Li and Reilly, 2008; Li *et al.*, 2008). Since qPCR generates amplicons as small as 60 bp (Bustin, 2002), the likelihood of fragmentation between priming regions is significantly reduced and thus yields more consistent results.
- (ii) It is important to ensure that data analyses are performed using samples of comparable RNA quality (Auer *et al.*, 2003; Fleige and Pfaffl, 2006). It is inappropriate to compare degraded and intact samples and thus necessitates a systematic RNA integrity control prior to any qPCR analysis (Bustin and Nolan, 2004; Imbeaud *et al.*, 2005; Pérez-Novo *et al.*, 2005).
- (iii) Special attention should be given to the extent of gene expression differences. Although large differences in gene expression may be reliably detected and quantified, even with partially degraded input RNA, small expression differences from low-quality RNA samples are prone to misinterpretation (Pérez-Novo *et al.*, 2005; Kerman *et al.*, 2006). For example, Koppelkamm *et al.* (2011), working with post-mortem human tissues, determined the sensitivity of the approach by means of a threshold of 7-fold change for a particular assay, below which the changes could not be distinguished from differences caused by impaired integrity. This permitted the authors to conclude that cases of suspected or detected RNA degradation require systematic validation of degradation profiles for all transcripts of interest in order to reveal detection limits of assays.
- (iv) From an experimental point of view, one of the more practical approaches is the 3'-based amplification method. The oligo(dT) priming method for cDNA synthesis will yield cDNAs that extend from the 3' end to the 5' end of mRNA, or to the cleavage site in the case of RNA degradation, which can be used for the more reliable detection of gene expression by targeting against 3' regions of the corresponding genes, an essential priming strategy for reverse transcription in microarray experiments. Not surprisingly, some gene expression profiling studies have shown tolerance to degraded RNA samples (Lee *et al.*, 2005): the Affymetrix GeneChip design, which is 3' biased, shows oligonucleotide probes that are usually designed to be within the last 600 nucleotides of the mRNA end (Li and Reilly, 2008). It is of particular interest that Opitz *et al.* (2010) have not observed a global effect of RNA quality on gene expression, stating that RNA degradation had a significant influence only on a small number of genes. Interestingly, the relative positions of probes from these genes in the corresponding cDNA sequences were shifted to the 5' region, while probes of the rest of normally represented genes were closer to the 3' end. This may allow for a noise reduction strategy by limiting

microarray analysis to probe sets closest to the 3' end of the transcripts (Turchin, 2006). Similarly, statistically significant differences have not been observed in expression levels between intact and degraded RNA samples of two target templates designed within ~800 nucleotides of the 3' end of the transcript using qRT-PCR, suggesting a high confidence region associated with the 3' end that may be utilized through careful primer design in quantifying mRNA levels (Die *et al.*, 2011). The approach's relevance is limited by the choice of oligo(dT) priming methods for cDNA synthesis, as well as the availability of information in public databases regarding gene structures.

## RNA quality in MIQE guidelines

There is an increasing consensus within the scientific community that the need to strengthen published information with relevant qPCR experimental detail is urgent (Huggett and Bustin, 2011). While guidelines that define the minimum information required for interpretation of microarray data have been available since 2001 (Brazma *et al.*, 2001), similar specifications for qPCR experiments have been more recently developed. In 2009, a set of qPCR best-practice guidelines was published by an international consortium of leading qPCR scientists, establishing the MIQE guidelines (Bustin *et al.*, 2009). Among the essential items to be reported, the quality assessment of RNA templates holds a prominent position. These guidelines are not a set of dogmatic principles; they instead provide a technical, common-sense approach for enhancing the reproducibility and transparency of qPCR data. Although adherence to the MIQE guidelines is not explicitly required by most leading journals (including those in the plant field), there has been an overall positive response to MIQE from researchers and authors (Bustin *et al.*, 2011), with >460 citations in the peer-reviewed literature since 2011. It is evident from the papers surveyed for this review that some areas of the pre-analysis qPCR steps require reformulation. Without providing information on RNA quality it is difficult to evaluate, as readers or reviewers, the relevance of any other reported data; this lack of transparency makes it difficult to reproduce experiments in different laboratories. A set of MIQE key parameters was recently reported in 2010, underlining the need to record RNA quality measurement once again. Although no perfect assessment method is postulated, microfluidics-based systems or 3':5' ratio assays are defined as basic indicators of RNA integrity (Bustin *et al.*, 2010).

## Conclusion

Analyses on a transcriptome-wide level using RNA-seq, or next-generation sequencing will undoubtedly transform transcriptomic biological research similarly to how the development of microarrays or the RT-qPCR technique changed the possibilities of mRNA quantification more than a decade ago. However, qPCR is clearly the most cost- and time-effective method currently available for a broad range of applications. The future of the technique most probably includes the standardization of practices and transparency in reporting data. An increasing number of

studies are dealing with recommendations for refining RT-qPCR standards. There are abundant examples where it is difficult to collect sufficient samples that meet the minimum quality threshold from the biomedical research. Despite some exceptions, plant molecular biologist should make use of the opportunity offered by the easier procurement of samples. Samples of the highest quality RNA possible, as well as those methods required to assess the critical issue of RNA quality, must be mandatory and freely accessible to revisers and colleges. This will help to maintain the level of quality and high standard of both works and publications.

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