Quantification of mRNA using real-time RT-PCR

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The real-time reverse transcription polymerase chain reaction (RT-qPCR) addresses the evident requirement for quantitative data analysis in molecular medicine, biotechnology, microbiology and diagnostics and has become the method of choice for the quantification of mRNA. Although it is often described as a "gold" standard, it is far from being a standard assay. The significant problems caused by variability of RNA templates, assay designs and protocols, as well as inappropriate data normalization and inconsistent data analysis, are widely known but also widely disregarded. As a first step towards standardization, we describe a series of RT-qPCR protocols that illustrate the essential technical steps required to generate quantitative data that are reliable and reproducible. We would like to emphasize, however, that RT-qPCR data constitute only a snapshot of information regarding the quantity of a given transcript in a cell or tissue. Any assessment of the biological consequences of variable mRNA levels must include additional information regarding regulatory RNAs, protein levels and protein activity. The entire protocol described here, encompassing all stages from initial assay design to reliable qPCR data analysis, requires approximately 15 h.

INTRODUCTION

RT-qPCR is a combination of three steps: (i) the reverse transcriptase (RT)-dependent conversion of RNA into cDNA, (ii) the amplification of the cDNA using the PCR and (iii) the detection and quantification of amplification products in real time[1]. Although it has become the method of choice for the quantification of RNA[2,3], there are several concerns that have a direct impact on the reliability of the assay. Principally, these are that (i) results depend on template quantity, quality and optimal assay design[4], (ii) the reverse transcription reaction is not standardized, hence can be very variable[5,6] and (iii) data analysis is highly subjective and, if carried out inappropriately, obfuscates the actual results obtained from the assay[7]. Consequently, it is essential to minimize variability and maximize reproducibility by quality-assessing every component of the RT-qPCR assay and adhering to common guidelines for data analysis. This clear need for standardization of gene expression measurements is particularly relevant in relation to human clinical diagnostic assays[8,9]. Consequently, we focused on quality control throughout the guidelines described in this protocol, so as to make it applicable to the development of standard operating procedures.

The assay

Real-time PCR (qPCR) uses fluorescent reporter dyes to combine the amplification and detection steps of the PCR reaction in a single tube format[10,11]. The assay relies on measuring the increase in fluorescent signal, which is proportional to the amount of DNA produced during each PCR cycle. Furthermore, the use of probes labeled with different reporter dyes allows the detection and quantification of multiple target genes in a single (multiplex) reaction. Individual reactions are characterized by the PCR cycle at which fluorescence first rises above a defined or threshold background fluorescence, a parameter known as the threshold cycle (C_t) or crossing point (C_p). The more target there is in the starting material, the lower the C_t. This correlation between fluorescence and amount of amplified product permits accurate quantification of target molecules over a wide dynamic range, while retaining the sensitivity and specificity of conventional end-point PCR assays. The closed-tube (homogeneous) format eliminates the need for post-amplification manipulation and significantly reduces hands-on time and the risk of contamination.

Current problems

The widespread use of this technology has resulted in the development of numerous protocols that generate quantitative data using (i) fresh, frozen or archival FFPE (formalin-fixed, paraffin-embedded) samples, (ii) whole-tissue biopsies, microdissected samples, single cells, tissue culture cells, (iii) total or mRNA, (iv) a range of different cDNA priming strategies, (v) different enzymes or enzyme combinations, (vi) assays of variable efficiency, sensitivity and robustness, (vii) diverse detection chemistries, reaction conditions, thermal cyclers and (viii) individual analysis and reporting methods. This obvious lack of standardization at every step of the assay (**Fig. 1**) is exacerbated by significant differences in sample processing, use of controls, normalization methods and quality control management[12] and has serious implications for the reliability, relevance and reproducibility of RT-qPCR[4,7,13].

Ideally, the uncertainty introduced at each step would be addressed by comparison of all available protocols. Since this is clearly impossible, we present a series of protocols that are based on current thinking for each of the steps included. Detailed discussions of sample selection, storage and RNA extraction are beyond the scope of this paper and are described at length elsewhere[14]. We begin this protocol with the validation of extracted RNA. We believe these protocols to be accessible to most researchers and to produce reliable data. In each case the decision to recommend a particular approach is justified, but distinct applications may require a variety of modifications that will be user-specific. An overview of the considerations relating to procedures and alternative steps for carrying out the RT-qPCR reaction is shown in **Figure 2**.

RNA assessment

A variety of procedures are in common use for quantification of RNA[15]. Students at the annual EMBO qPCR course (http://www-db.embl.de/jss/EmblGroupsOrg/conf_28) routinely compare

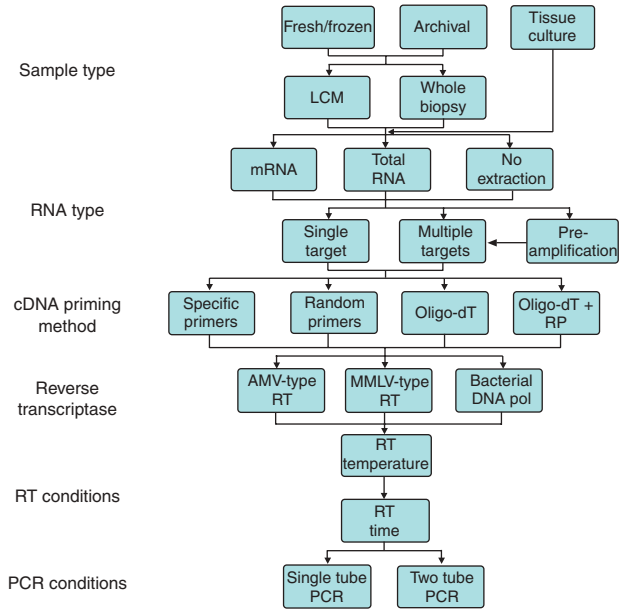

Figure 1 | Steps involved in planning a RT-qPCR assay. The numerous permutations illustrate the alternatives and potential for variability associated with this technique.

the use of Ribogreen, Agilent BioAnalyser, spectrophotometer, Nanodrop and more recently the BioRad Experion for quantification of the same RNA samples[12]. The results clearly demonstrate that no two methods produce the same data and that it is inadvisable to compare quantification data obtained using the different methods. This leads to the conclusion that it is prudent to measure all samples using the same technique and to report this information.

RNA quality

RNA quality encompasses both its purity (absence of protein and DNA contamination, absence of inhibitors) and its integrity. Traditionally RNA quality has been determined by analysis of the A260/A280 ratio and/or analysis of the rRNA bands on agarose gels, an approach updated with the introduction of the Agilent Bioanalyser/BioRad Experion microfluidic capillary electrophoresis systems. A recent report claims that it is possible to calculate a more objective measure of RNA quality by measuring several characteristics of the electropherogram generated by the Agilent 2100, including the fraction of the area in the region of 18S and 28S RNA, the height of the 28S peak, the fast area ratio and marker height and use these to calculate and assign an RNA Integrity Number (RIN) to each RNA sample[16]. However, an exhaustive analysis of the influence of RNA integrity on RT-qPCR assay performance comes to a different conclusion[15]. The authors extracted RNA samples extracted from numerous tissue types, subjected them to controlled degradation and analyzed them using the Agilent Bioanalyser. The samples had RINs between 10 (apparently intact material) to 4 (almost no evidence of rRNA bands). The quantity of individual transcripts in each of these samples was then determined using RT-qPCR assays. In some tissues the quantity of the measured transcripts detected was unaffected by the RIN whereas in others there was a linear relationship and in others a threshold response. Critically, the relationship between transcript quantity and RIN was different for different tissues and there was not a predictable relationship between these factors. The authors conclude that moderately degraded RNA samples can be reliably analyzed and quantitated, as long as the amplicons are kept short (<250 bp) and expression is normalized against an internal reference. The discrepancy between the two reports could be due to the relatively poor correlation coefficent (0.52) between RIN and expression values of the reference genes reported by the authors advocating the use of RINs[16].

In the absence of an alternative reliable measure of mRNA integrity, we propose the use of a 3′:5′ assay using GAPDH (NM_002046) as the target sequence (**Fig. 3**). The data obtained are independent of ribosomal RNA integrity, provide a reasonable measure of the degradation of the transcripts of interest and are modeled on the standard approach adopted by microarray users and long accepted conventional techniques applied to end point PCR assays for many years[17]. The 3′:5′ assay measures the integrity of the ubiquitously expressed mRNA specified by the GAPDH gene, which is taken as representative of the integrity of all mRNAs in a given 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 1.3 kb GAPDH mRNA is primed using oligo-dT, and a separate multiplex PCR assay is used to quantitate the levels of three target amplicons. These are spatially separated with one

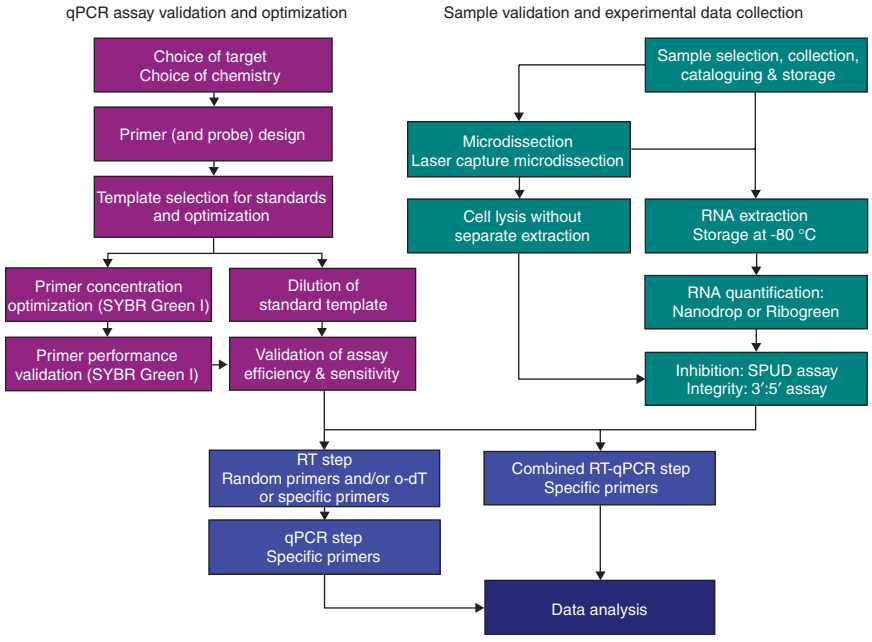

Figure 2 | RT-qPCR experimental workflow. The four main themes (assay validation and optimization, sample validation and data collection, RT-PCR assay and data analysis) are highlighted in different colors.

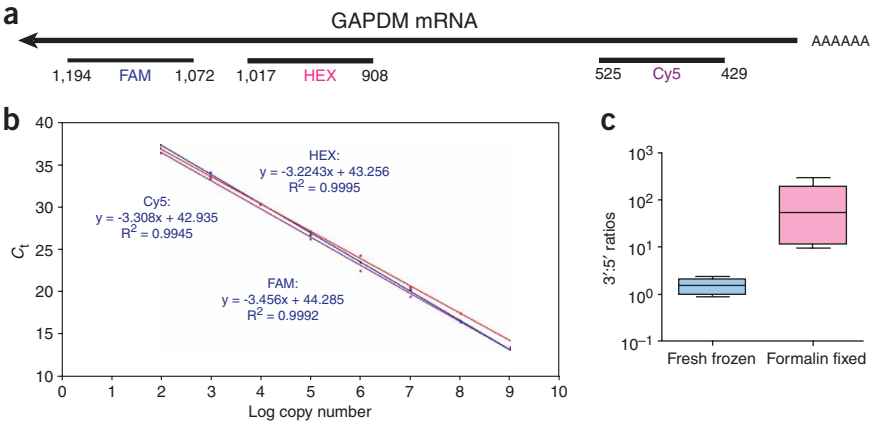

Figure 3 | The 3′:5′ assay used to estimate mRNA integrity. (**a**) Three sets of primers and probe target amplicons at different positions on the GAPDH (NM_002046) mRNA. (**b**) When run in a multiplex reaction, it is apparent that the amplification efficiencies of the three reactions are approximately the same, with similar y-intercepts indicating roughly similar sensitivity. (**c**) Difference in integrity between RNA extracted from fresh colonic biopsies on the left and RNA extracted from formalin-fixed, paraffin-embedded (FFPE) material on the right. Perfectly intact mRNA should result in a 3′:5′ ratio of 1–5, with denatured mRNA resulting in ratios of 10 or above. The median 3′:5′ ratio for GAPDH for the fresh biopsies is 2, indicative of high quality mRNA, whereas the median ratio for the FFPE biopsies is 90.

towards the 5′ end, the second towards the center 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. Clearly, the progress of the RT enzyme past the 5′ amplicon is dependent on the intactness of the mRNA, with the enzyme unable to reach it if the 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 (i.e., each amplicon is detected by a target-specific differentially labeled probe).

Inhibitory components

Inhibitory components frequently found in biological samples can result in a significant reduction in the sensitivity and kinetics of qPCR[18–23]. 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. At best, inhibitors can generate inaccurate quantitative results; at worst a high degree of inhibition will create false-negative results. 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 expression levels. However, this approach assumes that the two assays are inhibited to the same degree. The problem is even more pronounced in "absolute" quantification, where an external calibration curve is used to calculate the number of transcripts in the test samples, an approach that is commonly adopted for quantification of pathogens. Some, or all, of the biological samples may contain inhibitors that are not present in the nucleic acid samples used to construct the calibration curve, leading to an underestimation of the mRNA levels in the test samples[24]. The increasing interest in extracting nucleic acids from formalin-fixed paraffin-embedded (FFPE) archival material will undoubtedly lead to an exacerbation of this problem. Obviously, such inhibitors are likely to distort any comparative quantitative data. However, a recent survey of practices has revealed that only 6% of researchers test their nucleic acid samples for the presence of inhibitors[12].

Various methods can be used to assess the presence of inhibitors within biological samples. The PCR efficiency in a test sample can be assessed by serial dilution of the sample[25], although this is impossible when using very small amounts of RNA extracted, for example, from single cells or from laser capture microdissected sections. Furthermore, there are mathematical algorithms that provide a measure of PCR efficiency from analysis of the amplification response curves[26–28]. Internal amplification controls (IAC) that co-purify and co-amplify with the target nucleic acid can detect inhibitors as well as indicate template loss during processing[29]. Another approach utilizes a whole bacterial genome to detect inhibition from clinical samples[30]. We have recently described a qPCR reference assay, which we have named SPUD[31]. It identifies inhibitors of the reverse transcription or PCR steps by recording the C_ts 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 probe (SPUD P) (**Table 1**). In the presence of water, a C_t is recorded that is characteristic of an uninhibited reaction (e.g., around 25). 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) in the presence of sample RNA. Potential inhibitors in the RNA sample will result in a shift to higher C_t for these reactions when compared to those where RNA is absent.

Reverse transcription

The RT-qPCR assay can be performed either as a one-tube single RT and PCR enzyme method or a separate RT and PCR enzyme technique using one or a two tubes. The choices of reverse transcription enzyme and cDNA priming strategy are well debated[4–6] and are discussed in detail elsewhere[14]. Since reverse transcription yields depend on the target and the choice of reverse transcriptase, it is important to use the same enzyme, priming strategy and experimental conditions if results are to be comparable between laboratories[6]. cDNA priming strategy was the subject of an ABRF investigation in 2004/2005 (http://www.abrf.org/index.cfm/group.show/NucleicAcids.32.htm#R_4), and each approach has advantages and disadvantages. Since there is no one strategy that always works best, we have included protocols using random primers, oligo-dT and gene-specific primers in the RT section.

Single RT and PCR enzyme

A single enzyme such as *Tth* polymerase is able to function both as an RNA- and DNA-dependent DNA polymerase and can be used in a single tube without secondary additions to the reaction mix[32,33].

TABLE 1 | Oligonucleotide sequences for RNA integrity and inhibition detection assays.

	Oligonucleotides (5′–3′)	Stock concentration
5′-GAPDH (FAM)	P: CCTCAAGATCATCAGCAATGCCTCCTG	5 μM
	F: GTGAACCATGAGAAGTATGACAAC	10 μM
	R: CATGAGTCCTTCCACGATACC	10 μM
Center GAPDH (HEX)	P: CCTGGTATGACAACGAATTTGGCTACAGC	5 μM
	F: TCAACGACCACTTTGTCAAGC	10 μM
	R: CCAGGGGTCTTACTCCTTGG	10 μM
3′-GAPDH (CY5)	P: CCCACCACACTGAATCTCCCCTCCT	5 μM
	F: AGTCCCTGCCACACTCAG	10 μM
	R: TACTTTATTGATGGTACATGACAAGG	10 μM
SPUD amplicon	AACTTGGCTTTAATGGACCTCCAATTTTGAGTGTGCACAAGCTATGGAACACCACGTAAGACATAAAACGGCCACATATG GTGCCATGTAAGGATGAATGT	5 μM
SPUD (FAM)	P: TGCACAAGCTATGGAACACCACGT	5 μM
	F: AACTTGGCTTTAATGGACCTCCA	10 μM
	R: ACATTCATCCTTACATGGCACCA	10 μM

P, dual labeled or TaqMan Probe (HPLC purified); F, forward primer (desalt purified); R, reverse primer (desalt purified).

Its main advantages are the reduced hands-on time and potential for contamination. There are several disadvantages. First, since all reagents are added to the reaction tube at the beginning of the reaction, it is not possible to optimize separately the two reactions. Second, the assay can only be carried out using target-specific primers. Third, the assay may be less sensitive, due possibly to the less efficient RT-activity of *Tth* polymerase[34]. And fourth, the most thorough study comparing the two procedures found that this reaction is characterized by extensive accumulation of primer dimers, which may obscure the true results in quantitative assays[35]. We find that reducing the denaturation temperature to 92 °C and designing amplicons to be as short as possible can lessen some of these problems. This may be because this enzyme is somewhat less heat-stable than *Taq* polymerase.

Separate RT and PCR enzymes

Reactions can be either "coupled" (single tube) or "uncoupled" (two tubes). In the "coupled" alternative the reverse transcriptase synthesizes cDNA in the presence of high concentrations of dNTPs and either target-specific or oligo-dT primers. Following the RT reaction, PCR buffer (without Mg^{2+}), a thermostable DNA polymerase and target-specific primers are added and the PCR is performed in the same tube. In the "uncoupled" alternative, the reverse transcriptase synthesizes cDNA in a first tube, under optimal conditions, using random, oligo-dT or sequence-specific primers. An aliquot of the RT reaction is then transferred to another tube containing the thermostable DNA polymerase, DNA polymerase buffer, and PCR primers and the PCR is carried out under conditions that are optimal for the DNA polymerase. Interassay variation of two enzyme protocols can be very small when carried out properly, with correlation coefficients ranging between 0.974 and 0.988[35]. The disadvantages of this approach are (i) in two-enzyme/one-tube assays, a template switching activity of viral RTs can generate artifacts during transcription[36] and (ii) there are additional opportunities for contamination and the RT enzyme can inhibit the PCR assay even after inactivation, resulting in an overestimation of amplification efficiency and target quantification[37].

On balance, however, for research applications the increased flexibility, sensitivity and potential for optimization makes the use of a two-enzyme protocol preferable to the single-enzyme procedure. For diagnostic purposes, the reduced hands-on time and potential for contamination may make the one-tube/enzyme approach more attractive.

cDNA priming

Random priming or oligo-dT priming both allow a representative pool of cDNA to be produced during a single reaction. However, it has been shown that priming using random hexamer primers does not result in equal efficiencies of reverse transcription for all targets in the sample and that there is not a linear correlation between input target amount and cDNA yield when specific targets are measured[4,38]. A recent comparison of the efficiency of RT priming by random primers of varying lengths showed that 15-nt-long random oligonucleotides consistently yielded at least twice the amount of cDNA as random hexamers[39]. The 15-mers were more efficient at priming, resulting in reverse transcription of greater than 80% of the template, while random hexamers induced reverse transcription of only 40%. Not surprisingly, this resulted in the detection of one order of magnitude more genes in whole transcriptome DNA microarray experiments. Consequently, we would suggest the use of 15-mers for random-primed cDNA synthesis reaction. However, since the authors did not address the question of linearity it remains to be seen whether this change overcomes that particular limitation of the random hexamer approach.

Oligo-dT primers should only be used with intact RNA. Even then the cDNA molecules may be truncated, since the RT enzyme cannot proceed efficiently through highly structured regions. Accordingly, oligo-dT-primed assays should be targeted towards the 3′ end of the transcript. This is an unsuitable choice for experiments that require examination of splice variants, sequences

with long 3′ UTR regions or those without polyA sequences. Furthermore, oligo-dT priming is not recommended when using RNA extracted from paraffin tissue sections, since formalin fixation results in the loss of the polyA tails on mRNA[40].

Target specific primers are the most specific and most sensitive method for converting mRNA into cDNA[41] and are the recommended choice when RNA quantity is not a limiting factor. Even then, a recent report demonstrates the use of specific primers for the reliable and specific amplification of 72 genes from limiting amounts of RNA using a multiplexed tandem PCR approach[42]. Nevertheless, as with random priming there may be differences in the efficiencies at which individual RT reactions occur. These variations must be controlled for by reference of unknown samples to a calibrator sample (when using $\Delta\Delta C_t$ analysis) or to standard curves. Specific priming of RNA dilutions results in a linear response of target cDNA yield[4] and so a further advantage of using this priming method is that the efficiency of the combined RT-qPCR reaction can be confirmed by analysis of the gradient of the standard curve. The inclusion of a calibrator sample or standard curve on every plate is an important control for measuring inter-assay variability that may occur when multiple samples are run on different plates.

PCR optimization

Assay optimization is the key to better sensitivity, specificity, reproducibility and a wider linear dynamic range. Fortunately, whereas the RT step is highly variable, the qPCR segment of the assay is remarkably reproducible when run under optimal conditions, although greater variability is observed between replicates when the assay is run under suboptimal conditions[43].

The thermodynamic stability (ΔG) of a duplexed primer/target structure differs for different primers and varies with primer concentration. Therefore, it is important to use primers at concentrations that result in optimal hybridization and priming. Although optimization of PCR reactions used to be an essential part of assay development, recent trends towards high throughput and rapid data reporting have resulted in the elimination of this step in many laboratories. Indeed, some manufacturers claim it is not necessary to optimize primer concentrations when using their particular mastermixes. However, it is clear that the rationale underlying the original recommendations remain valid and that optimization of primer concentration can significantly improve assay detection sensitivity (**Fig. 4**).

We suggest the use of a fluorescent nucleic acid dye such as SYBR Green I or EvaGreen, together with an analysis of amplification plots and melt curves to optimize assays. Melt curves are a powerful means of providing accurate identification of amplified products and distinguishing them from primer dimers and other small amplification artifacts. DNA melts at a characteristic temperature called the melting temperature (T_m), defined as the temperature where half of the DNA helical structure is lost. The melting temperature of a DNA molecule depends on both its size and its nucleotide composition; hence GC-rich amplicons have a higher T_m than those having an abundance of AT base pairs. During melt curve analysis, the real-time machine continuously monitors the fluorescence of each sample as it is slowly heated from a user-defined temperature below the T_m of the products to a temperature above their melting point. Fluorescent dye is released upon melting (denaturation) of the double-stranded DNA, providing accurate T_m data for every single amplified product. Melting peaks are calculated by taking the differential (the first negative derivative (–dF/dT) of the melt curve. These peaks are analogous to the bands on an electrophoresis gel and allow for the qualitative monitoring of products at the end of a run. Short primer dimers will melt at lower temperature than longer, target amplicon products.

Conclusions

We advocate that the following procedures are routinely implemented to ensure optimal reproducibility. Their universal adoption and inclusion in published reports would constitute considerable progress towards the development of a standardized procedure that would reduce the uncertainty surrounding data from different studies. (i) Appropriate sample selection when quantitating mRNA levels from biopsies is a basic criterion for obtaining biologically meaningful data. We strongly advise the use of microdissection for relating quantification and localization of expression data. (ii) An assessment of the quality of any RNA preparation used

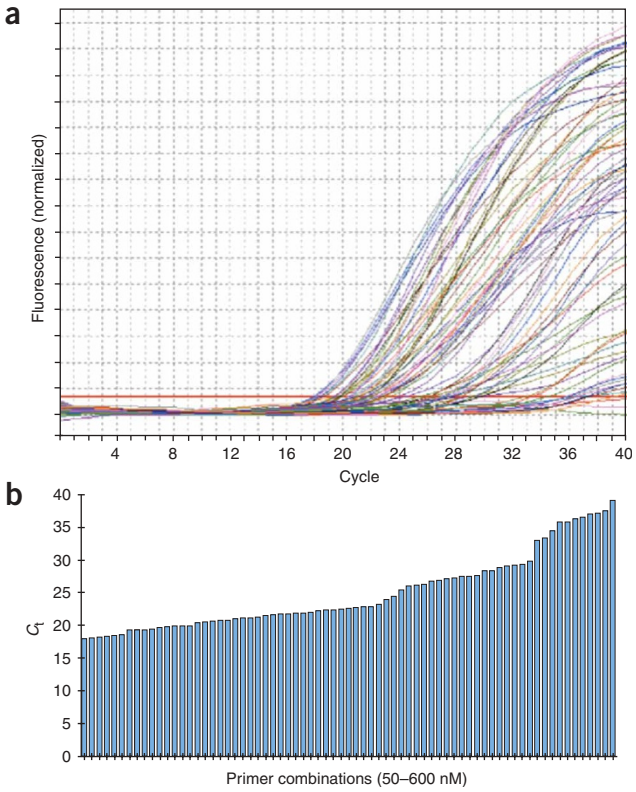

Figure 4 | Optimization of primer concentration. cDNA derived from total RNA (mouse) was amplified using PCR primers specific to Hepcadin 1 (ref. 62). Since primer conditions were not specified, all combinations of forward and reverse primer concentrations ranging between 50 nM and 600 nM were used, similar to the abridged mismatched primer concentration matrix (**Table 2**). (**a**) The amplification plots reveal the huge differences in C_t, slope and plateau obtained using identical templates. Each amplification plot represents a different combination of primer concentration. The horizontal red line represents the threshold, which was set automatically by the instrument. (**b**) The different primer concentrations resulted in a C_t range of more than 20 cycles with low concentrations of the forward primer corresponding to high C_t values. The most appropriate primer combination is the one that gives the lowest C_t and the highest normalized fluorescence.

BOX 1 | CONSIDERATIONS FOR RT-qPCR PROCEDURES

General
- For all procedures use DNase/RNase-free consumables.
- Maintain a dedicated set of micropipettes and use filter barrier tips for all qPCR reactions.
- Dilute the template so that between 3 μl and 10 μl are added to each qPCR reaction. This reduces inaccuracies due to attempting to pipette very low volumes.
- Always aliquot all reaction components and use fresh aliquots if product is detected in the no template control (NTC).
- Always include a NTC after all reagents have been dispensed to reveal potential cross contamination.
- Defrost all reagents on ice and mix well prior to making up reaction mixes (we prefer inversion to vortexing, followed by a quick spin).
- Avoid exposing fluorescent probes and fluorescent nucleic acid binding dyes to light.
- When preparing mastermixes take care to ensure that all samples and controls have been accounted for. Make extra mix to allow for pipetting irregularities (usually around 10% or an extra sample is sufficient).
- When using a block-based PCR system briefly spin reaction tubes to ensure the removal of bubbles.

Reverse transcription
- In general, use total RNA as the template for the reverse transcription.
- Add the same amount of total RNA into each reaction.
- When possible use target specific primers; if using random primers use 15-mers[39].
- Use a thermostable RT enzyme such as Superscript III.
- If possible process samples simultaneously to avoid batch-to-batch variation.
- Always include no-RT controls to reveal the presence of contaminating gDNA. This is important if the target transcript is present in low copy numbers, where the assay cannot be designed across intron-exon boundaries or where the intron sequences are less than 1 kb and so might amplify during the PCR step.

PCR
- Positive template control (PCR product, synthetic amplicon, linearized plasmid) to check for consistency of reaction.
- A NTC (i.e., water control for PCR) is essential to check for non-specific signal arising from primer dimers or template contamination.
- Perform reactions in duplicate (triplicate if C_ts are >35). If the data from these differ by >0.5 C_t, the reactions should be repeated. If the reproducibility is consistently low, the assay should be re-optimized. Carrying out reactions in duplicate is generally sufficient. It is more important to run biological duplicates, as these will identify the true variability within the data.

for quantification is essential. In particular, in all publications, information should be provided regarding the absence of inhibitors in each sample and, for cellular RNA, the integrity of the mRNA. (iii) The quantity of RNA should be determined using a single method and the amount used for the RT step must be kept as constant as possible. (iv) The cDNA priming method must be consistent, with specific priming the preferred option, together with constant timing (as short as possible) and reaction temperature (as high as possible). (v) Normalization must be transparent and internal reference genes, if used, must be validated for each experimental set-up and appropriate data should be included in every publication. (vi) Amplification of target-specific standard curves should be analyzed and data reported to reveal the amplification efficiency and sensitivity of every PCR assay, and to confirm that the quantification of any unknowns has been carried out within the dynamic range of that assay. (vii) Any signal detected in the negative controls should be reported and qualified by melt curve or other analysis. (viii) When reporting data as a relative change (i.e., when using $\Delta\Delta C_t$ analysis) the C_t range of target detection should be quoted.

A more detailed discussion of these and other considerations can be found elsewhere[14] and online (http://www.gene-quantification.info/). Important practical considerations for all RT-qPCR procedures are outlined in **Box 1**.

MATERIALS

REAGENTS

- Commercial RNA extraction kit. For RNA extracted from formalin-fixed, paraffin-embedded (FFPE) material we recommend the use of the Absolutely RNA kit (Stratagene, cat. no. 400809-1). For RNA from fresh/frozen microdissected samples we recommend the RNeasy microkit (Qiagen cat. no. 7400). Alternatively, when dealing with very small amounts of sample (e.g., from single cells or when using minute amounts of laser capture microdissected fresh/frozen sample) it is more exact to carry out the RT-PCR directly from lysed tissue without going through an extraction process[44] and we have had good success with Invitrogen CellsDirect RT-qPCR kits (cat. no. 11753-100 (one tube) or 11737-030 (two tube)).
- RiboGreen RNA quantification assay (Molecular Probes/Invitrogen cat. no. R11490).
- DNase/RNase-free water (e.g., Ambion, cat. no. 9922/9924 or 9932/9934; Sigma Aldrich cat. no. W4502; Bioline cat. no. BIO-38030 or 38031)
- Universal RNA to use as control material or standard curve template (Stratagene cat. no. 750500 (human), 7410100 (mouse), 740200 (rat)).
- Oligonucleotides (see REAGENT SETUP). Oligos are available from numerous suppliers (e.g., Sigma-Genosys or Sigma-Proligo for LNA and BHQ modifications, and Eurogentec, Applied Biosystems or Epoch for MGB modified probes. The oligonucleotides used for RNA quality assessments described in this protocol are listed in **Table 1**.
- Detection assay (see REAGENT SETUP): SYBR Green I (e.g., Stratagene Brilliant SYBR Green RT-qPCR kit cat. no. 600552, Sigma Aldrich SYBR Green Jumpstart cat. no. S9194 or Qiagen QuantiTect SYBR Green RT-PCR kit cat. no. 204243), EvaGreen (Biotium cat. no. 3100) or TaqMan (e.g., Stratagene Brilliant Probe-based RT-qPCR kit cat. no. 600551, Sigma Aldrich Jumpstart cat. no. D7440 or Qiagen QuantiTect Probe RT-PCR kit cat. no. 204443). It is also possible to purchase a single kit containing all the components for either SYBR Green or probe-based assays

(e.g., Quantace SensiMix cat. no. QT205-02 (one tube)/QT305-02 (two-tube)).
- Polymerases: *Tth* polymerase (e.g., Applied Biosystems cat. no. N8080098; Roche cat. no. 11480014001) for single-tube procedure. For two-tube procedure, use a thermostable reverse transcriptase (e.g., Superscript III, Invitrogen cat. no. 18080-400 or eAMVTM, Sigma cat. no. A4464) and a hot start *Taq* polymerase plus Master Mix (e.g., Qiagen HotStarTaq cat. no. 203443; Invitrogen Platinum Taq cat. no. 10966)
- dNTP mix (e.g., Fermentas cat. no. R1121 (25 mM each) or R0192 (10 mM each); Bioline cat. no. BIO-390028 (25 mM each); Roche cat. no. 11581295001)
- TE buffer pH 7.0–8.0 ▲ CRITICAL if preparing your own TE-buffer, the pH must be adjusted before the EDTA will dissolve.
- Yeast tRNA (e.g., Invitrogen cat. no. 15401029 or Ambion cat. no. 7119) for dilution of RNA used to generate standard curve preparation. Use at 100 ng/μl.

EQUIPMENT
- Agilent 2100 Bioanalyser (cat. no. G2940CA) or BioRad Experion (cat. no. 700-7001 (100/120V); 700-7002 (220/240V)
- Nanodrop ND-1000 Spectrophotometer (for a small number of samples)
- A fluorescence plate reader, if the qPCR system (see below) cannot be used as a fluorescence sample reader.
- Filter barrier pipette tips (e.g., Axygen TF-series Maxymum recovery pipettor-specific tips)

REAGENT SETUP

Primer design resources Before designing an assay from scratch, it is worth checking whether any validated assays are available elsewhere. By this we mean assays that have been published in peer-reviewed publications and provide all the information with regard to efficiency, specificity, sensitivity and absence of primer dimers. This will not only forestall the re-invention of the wheel, but will also help with the objective of creating more standardized and uniform assays[45,46]. However, it is not a good idea to use any of the pre-designed assays offered by some commercial suppliers, unless they provide all relevant information, in particular data concerning the assay's actual performance. Even then, it is essential to validate the assay's performance in the end user's laboratory, particularly with respect to its amplification efficiency and sensitivity limits.

The best sources for primers and probes are public primer and probe databases such as RTPrimerDB (http://medgen.ugent.be/rtprimerdb/), PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) or Real Time PCR Primer Sets (http://www.realtimeprimers.org/). RTPrimerDB lists validated qPCR assays submitted by researchers and includes all the information required to understand the purpose of an assay and how to implement it in an experiment. PrimerBank lists more assays (180,000) but only a small percentage of the assays designed using uPrimer[47] have been practically validated. Real Time PCR Primer Sets lists additional validated hybridization probes and molecular beacons.

If none of these assays is suitable, there are numerous software packages available for PCR primer design, both free and commercial. When designing a new assay, appropriate quality control of that assay is essential, with a combination of the highest possible efficiency and sensitivity, together with the absence of primer dimers being the most important parameters. Probably the most comprehensive commercial program is Beacon Designer (http://www.premierbiosoft.com). When the number of assays to be designed does not justify the outlay for the program, design services run by suppliers can be used (e.g. Sigma-Genosys at http://www.designmyprobe.com).

An excellent alternative is the free, web-based primer/probe design program at http://www.biosearchtech.com/products/probe_design.asp. It is best to test four to six primers per amplicon in their various combinations, since this is most likely to identify the most efficient and sensitive assay. Once the assay is designed, the software directly links to the NCBI databases to enable sequence retrieval using accession numbers as well as BLAST searches. Using the link to NCBI's ePCR site, the specificity of any assay design can be confirmed via virtual PCR.

Primer design considerations DNA primers should be between 15 and 25 bases long to maximize specificity, with a G/C content of around 50%. This also contributes to a higher yield of full-length product after synthesis and purification. For primers binding at very AT-rich sequences, it is advantageous to substitute one or more of the bases with a locked nucleic acid (LNA) analog to reduce the overall primer length while maintaining the T_m. Avoid primers with secondary structure (i.e., inverted repeats) or with sequence complementarity at the 3′ ends that could form dimers. Due to competition between intermolecular (primer-template, probe-target) hybridization, and intramolecular hybridization, inverse repeats can cause inefficient priming and probing of the target sequence. PCR reaction and/or probe binding can completely fail because of formation of stable hairpins at the binding region, or inside the amplicon in general. Primer-dimers have a negative ΔG value, so primers should be chosen with a value no more negative than –10 kcal/mol. The 3′ terminal position is essential for the control of mispriming during the PCR reaction. Primers with one or more G or C residues at that position will have increased binding efficiency due to the stronger hydrogen bonding of G/C residues. It also helps to improve the efficiency of priming by minimizing any "breathing" that might occur. However, avoid clamping the 3′ of the primer because ambiguous binding of oligonucleotides to the target site could result in misprimed elongation ("slippage effect"). A reasonable comprise is to aim for no more than 2 or 3 G/C in the five 3′ bases of the primer. Similarly, avoid long runs of a single base (i.e., more than three or four, especially G or C) since homopolymeric runs can also cause the "slippage effect". In general, primer concentrations for fluorescent DNA binding dye assays are lower (around 50 nM) than those for probe-based assays (200–400 nM).

Probe design considerations qPCR assays using hydrolysis (TaqMan) probes are usually carried out as two-step reactions, with a denaturation step followed by a combined annealing/polymerization step, during which fluorescence is measured. Most detection systems rely upon binding of the probe to the template before hybridization of the primers. It is for this reason that the probe T_m should be around 10 °C higher than the T_m of the primers, usually in the 68–70 °C range. The probe should be designed with the 5′ end as close to the 3′ end of one of the primers as is possible without them overlapping. This ensures rapid cleavage by the polymerase. The probe should be no more than 30 nucleotides in length to maximize quenching, with a G/C content of around 50%. If the target sequence is AT-rich, incorporate analogs such as locked nucleic acids (LNA; Roche) or minor groove binders (MGB; Applied Biosystems). LNA is a nucleic acid analog in which the ribose moiety in the sugar-phosphate backbone is structurally constrained by a methylene bridge between the 2′-oxygen and the 4′-carbon atoms. The link 'locks' the sugar ring in the fixed 3′-endo conformation, which is preferable for the formation of hybrids with complementary DNA or RNA sequences. This feature gives the LNA probes very high binding affinity but does not compromise their sequence specificity. MGBs are dsDNA-binding agents that are attached to the 3′ end of TaqMan probes and also stabilize the hybridization reaction. In practice, this allows the design of shorter probes, which increases design flexibility. Guanine (G) can quench fluorescence and so is avoided as the 5′ base. In this position the G would continue to quench the reporter even after cleavage, resulting in reduced fluorescence values (ΔR), which could result in reduced sensitivity. Any runs of four or more identical nucleotides, especially G, should be avoided because these can affect the secondary structure of the probe and reduce hybridization efficiency. Where an alternative sequence cannot be selected, disruption of a series of Gs by the substitution of an inosine can significantly improve probe performance.

Oligonucleotides Oligonucleotides to be used for PCR priming should be ordered with desalt purification, those with fluorescent labels to be used as probes should be HPLC purified; long oligonucleotides used as amplicon target molecules require PAGE purification. For multiplexing, select different fluorescent labels as appropriate for the instrument used. It is important to avoid detection of nonspecific signal and so the wider the spectral range used the better. For example, a dualplex reaction is best carried out using FAM quenched by the dark quencher BHQ1 (BioSearch), and HEX quenched by BHQ1 or BHQ2 (Biosearch). ▲ CRITICAL Order primers, probes and amplicons at different times and ensure the manufacturer does not lyophilize them, since this will result in cross-contamination. Stock solutions should be prepared with DNase/RNase-free water and aliquotted to avoid whole-batch contamination and repeated freeze/thawing. Forward and reverse PCR primers should be stored at −20 °C, at 10 μM or as a stock at 10× working concentration. Probes should be protected from light by wrapping in foil and stored at −70 °C, either as lyophilized salt or as a 5 μM solution. Working solutions of primers and probes can be stored at 4 °C for up to 2 weeks. Long-term storage of stock solutions is variable, ranging from 6 months to several years.

Choice of detection assay The appropriate detection assay depends on the application. The use of a double-stranded DNA binding dye, typically SYBR Green I or EvaGreen, is the most cost-effective chemistry for initial investigations and for primer optimization steps. Although the primers alone determine the specificity of product detection, information regarding product size and

population is easily determined from a melt curve analysis. Other non-probe based assays include Amplifluor (http://www.Chemicon.com), Promega's Plexor (http://www.promega.com) and Invitrogen's LUX primer (http://www.invitrogen.com).

At very low (<1,000 copies) target concentration there is a greater probability of nonspecific amplification and problems with primer dimer products becoming more pronounced. In this case, the use of a probe to detect amplicons may be preferable, although note the use of Syto 9 fluorescent dye (Invitrogen cat. no. S34854) or EvaGreen (Biotium cat. no. 31000) and mt-PCR for multiplex analysis using melt curves[42]. The most widely used chemistry uses the dual-labeled fluorescent (TaqMan) probe, which can be synthesized with added locked nucleic acid (LNA) or minor groove binder (MGB) modifications. This is particularly useful when targeting AT-rich sequences and when targets are highly structured or repetitive.

A wide range of qPCR and qRT-PCR mastermixes are available. Capillary-based systems from Roche (LightCycler range) require the addition of BSA into the reaction buffer to prevent binding of reaction components and nucleic acid to the glass. Otherwise reaction mixes are largely interchangeable for most instruments. An important perceived advantage of some instruments and reagents is that they can be used to speed up the amplification process. However, although fast cycling conditions do not affect assay specificity, they can affect sensitivity and result in increased variability[43].

Considerations for reverse transcription (RT) reaction Since secondary structures in mRNA can cause the RT to skip over the secondary structure we recommend the use of a thermostable reverse transcriptase such as Superscript III (Invitrogen cat. no. 18080-400) or eAMVTM (Sigma cat. no. A4464). The RT step should be carried out at 60 °C for 15 min using the supplied 2× reaction mixture and amplicon-specific primers as this increases the specificity of the reaction, by minimizing false priming. Although we do not recommend their use without applying the necessary precautions[4], if using oligo-dT or random primers, cDNA priming must be initiated at a lower temperature. Whichever enzyme is used, the buffer supplied by the manufacturer is optimized for that enzyme and should always be used.

Considerations for PCR reaction There are two important considerations: first, when carrying out 5′-nuclease (TaqMan) assays there is an absolute requirement for a thermostable polymerase with a 5′–3′ nuclease activity and second, if targeting low copy number targets using fluorescent DNA binding dyes, the use of a hot start *Taq* polymerase is necessary, either complexed with an antibody or chemically modified to inhibit polymerase activity at low temperatures. We have used enzymes and kits from all the major suppliers and have found no major, consistent differences in quality but note that antibody inactivated enzymes require a shorter period of activation. Again it is important to use the buffer supplied with the enzyme, since the manufacturer has optimized it for that enzyme. Some manufacturers include PCR enhancer solutions in their kits. It is important to read the instructions carefully, since they can affect the reaction conditions. For example, if designed for amplification of G/C rich regions, they may lower the DNA melting temperature, thus lowering the primer annealing temperature. Combined reagent mastermixes are increasingly popular. These have the advantage of reducing potential errors when combining components prior to reactions. In many cases they are stabilized with components that will also disrupt secondary structures in templates and so can give improved results. In the case of difficult templates, a sequence specific effect with specific reagent blends will be observed and so trying a different blend may alleviate problems. They are provided in convenient format and suitable for routine or high throughput assays. The disadvantage is that there is less flexibility for optimization of challenging simplex or multiplex assays. Take care to check the mixture components with particular attention to matching $MgCl_2$ concentration to the detection chemistry used and the presence and concentration of reference dyes such as ROX.

▲ **CRITICAL** The following qPCR protocols are applicable for either commercial master mixes or reagents prepared in-house from individual components. Do check stock concentrations, as these can vary between suppliers. The reactions are quoted for 25 µl reactions since this is the most popular reaction volume, despite most manufacturers recommending 50 µl reactions. In some cases, especially with very low target copy numbers, the higher volume may give better reproducibility between samples; for most reactions, however, it makes no difference and reaction volumes as low as 1–5 µl have been reported, particularly when using a centrifugal rotary instrument such as the Corbett Rotorgene. If in doubt, perform a comparison experiment to establish if there are differences for a particular assay.

EQUIPMENT SETUP

Choice of real-time thermal cycler A recent publication compares the performance of three different instruments[48]. A comparison of the manufacturer supplied information can be obtained for selected instruments at http://www.biocompare.com. However, this is no substitute for testing the instrument and conferring with experienced users to gain an unbiased opinion. For multiplexing it is advisable to use halogen or LED-based instruments in preference to instruments using lasers, as this allows a more precise matching of emission spectra and fluorophores. Ease of use of software and data visibility are critical factors. Ensure raw data can be viewed and that there are user-controlled analysis options. Note, the qPCR instrument can be used for quantification of RNA using the Ribogreen assay. ▲ **CRITICAL** For multiplex experiments, instruments capable of detecting several fluorophores must be used.

Choice of PCR plastic ware Some systems are compatible with heat-sealed film coverings that work very well; however, it is essential to ensure that the seal is complete to avoid sample evaporation. If it is desirable to retrieve the sample after qPCR these seals can be pierced, thus avoiding well to well or work area contamination. It is inadvisable to remove 8 or 12 strip caps since this usually results in droplets of reaction being carried into nearby tubes as well as all over the immediate working area. If the sample is required post reaction use individual sealed tubes or film (as above) and open samples in a separate room. ▲ **CRITICAL** When a block system is used the plates must fit well to ensure efficient thermal transfer and uniformity between wells. This is also why it is important to use thin walled tubes. When signals are detected through the cap of the system ensure that optical grade caps are used. ▲ **CRITICAL** Avoid contamination by using dedicated sets of micropipettes for RT-PCR only, with one micropipette reserved for dispensing RNA. Pipettors must be calibrated regularly, especially those dispensing 10, 2, or 1 µl and it is essential to use the pipettor appropriate for the volume being dispensed.

PROCEDURE

Selection of suitable amplicon(s) from target gene sequence ● TIMING 30 min to 3 h

1| Check RTPrimerDB (http://medgen.ugent.be/rtprimerdb), PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) and Real Time PCR Primer Sets (http://www.realtimeprimers.org) for a suitable validated qPCR assays. If an assay is not found, design a new assay as follows using the criteria described under REAGENT SETUP.

2| Input the target nucleotide sequence into a BLASTn search using the default settings (http://www.ncbi.nlm.nih.gov/blast).

3| Examine the aligned sequences for polymorphisms and possible errors and avoid these regions for primer or probe design.

4| Avoid direct repeats in the target sequence; hybridization to alternative sites in repetitive regions results in non-productive binding of primers, a reduction in the efficiency of DNA amplification and a concomitant decrease in the sensitivity of the assay.

5| Consider potential splice variants and target the required amplicons appropriately. Where appropriate, identify intron-exon boundaries by comparing cDNA and genomic sequences and design assays across the longest intron region; this reduces the possibility of amplification from genomic DNA (gDNA) contaminants. This is essential when using genomic DNA for normalization or when targeting specific splice variants of an mRNA. It is most economical to place the downstream primer across the splice junction, as this allows the use of a single (expensive) probe for the detection of possible splice variants in separate

BOX 2 | DNASE I DIGEST

1. Dilute RNA to a concentration of approximately 50 ng/μl.
2. Prepare 10× DNase I buffer (100 mM *Tris* pH 7.5, 25 mM $MgCl_2$, 5 mM $CaCl_2$).
3. Carry out the DNase I digestion in a total volume of 100 μl using a maximum of 2 μg RNA, incubating for 30 min at 37 °C.
Note: It has been reported that the use of buffer containing Mg^{2+} for DNase I cleavage of DNA contaminants in RNA samples leads to DNA artifacts after RT-PCR. These are absent if Mn^{2+} buffer (1 mM final concentration) is substituted[61]. NaCl is preferentially used as the source of monovalent cation to avoid precipitation of SDS that may be present after purification. Alternatively NaOAc can be used at the same concentration and is the preferred salt when dNTPs may be present in the RNA preparation.
4. At this stage, the RNA can be stored (option A) or purified further by phenol extraction (option B).

(A) Storage

(i) Place tubes for 10 min at 75 °C
(ii) Quick freeze and store at –80 °C.

(B) Phenol extraction

(i) Add 100 μl of acid phenol/chloroform (pH 6.6) to the DNase I digest.
(ii) Vortex twice for 15 s and centrifuge at top speed in a refrigerated microfuge at 5 °C for 2 min.
(iii) Transfer the aqueous (upper) phase to a new tube.
(iv) Back-extract the organic phase with 50 μl of TE buffer.
(v) Repeat Step iii and combine the two aqueous phases.
(vi) Add 15 μl 3M NaCl and 300 μl of 100% ethanol. Mix well.
(vii) Store for 15 min in dry ice/EtOH bath.
(viii) Centrifuge in refrigerated microfuge at 5 °C at top speed for 15 min. Discard supernatant.
(ix) Cover the pellet with a layer of 500 μl of 70% EtOH. Vortex to resuspend pellet.
(x) Centrifuge in refrigerated microfuge at 5 °C at top speed for 15 min. Discard supernatant.
(xi) Air-dry the pellet. Resuspend the RNA in 50 μl of TE-buffer.

assays. However, if the choice is between an inefficient and/or insensitive assay designed across a splice junction and an efficient one contained within a single exon, it is better to use the latter. In this case, it is advisable to treat the sample with DNase I (see Step 9 and **Box 2**) and essential to add a 'no RT' control to determine the contribution of any DNA contamination to the mRNA quantification. Since this control contains all the ingredients except for the RT enzyme, any subsequent amplification will be derived from contaminating gDNA present in the RNA sample.

6| Check target sequence folding at the temperature used for the RT step (http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi) and avoid highly structured regions where hybridization of primers or probes to the target will be inefficient.

7| Aim for as short an amplicon as possible (within 60–150 bp) with a G/C content of 60% or less to ensure efficient denaturation occurs during thermal cycling, leading to a more efficient reaction. GC-rich sequences are also susceptible to nonspecific interactions that result in nonspecific signal in assays utilizing fluorescent nucleic acid binding dyes. Small amplicons require shorter polymerization times for replication, making amplification of gDNA contaminants less likely. It is also easier and cheaper to synthesize a shorter oligonucleotide if a synthetic sense-strand oligonucleotide is used to generate amplicon-specific standard curves (see Steps 38–43). If using oligo-dT priming, design the amplicon towards the 3′ region of the template.

▲ **CRITICAL STEP** For multiplex reactions, design all assays together (Beacon Designer will automatically design up to four assays to be run in multiplex). Maintain the same amplicon lengths (within 5 bp) and annealing temperatures for oligonucleotides. Check that there is no potential for the oligonucleotides to cross-hybridize between reactions.

RNA extraction ● TIMING 30 min

8| Extract RNA using a commercial RNA extraction system, following the manufacturers' instructions precisely. In our experience there is little difference between the kits provided by the main suppliers (Qiagen, Ambion, Stratagene, Promega, Sigma). Tri-reagent based protocols (e.g., Sigma Aldrich cat. no. T9424 or Invitrogen cat. no. 15596-02) tend to provide a better yield than column-based methods, although there is a potential for co-purifying phenolic compounds that can inhibit downstream reactions. In our hands, the most consistent results are obtained using column-based protocols (e.g., Qiagen's RNeasy Plus Mini kit, cat. no. 74134) in conjunction with an on-column digestion step with DNAse I.

9| Consider additional DNAse I treatment if the target gene is intron-less or if the primers span short introns, since any contaminating DNA is likely to be co-amplified and could interfere with accurate quantification. The simplest solution is to use Ambion's DNase-free DNA Treatment and Removal reagent (cat. no. 1906). Less expensive, but more likely to result in loss of RNA and potential phenolic contamination is to DNase-I treat and then remove the enzyme using a phenol extraction followed

by ethanol precipitation (see **Box 2**). It has also been suggested that a 10 min incubation at 75 °C is sufficient to destroy all DNase I activity and preserve 100% of RNA integrity[49]; however, we have not tested this approach. **! CAUTION** Do not accept on faith any manufacturer's claim that their reagent is RNase-free[50]. Always test the DNase I on an expendable sample and use as little DNase I for as short a time as possible.

Quantification of RNA

10| Quantify RNA using an appropriate method. The Nanodrop spectrophotometer (Option A) is the preferred method when quantitating up to ten samples at any one time. RiboGreen (Option B) is the most accurate and convenient means of quantitating large numbers (>10) of RNA preparations and is best carried out in a 96 or 384 well plate. Ribogreen is a fluorescent nucleic acid stain. The bound complex has an excitation maximum of around 500 nm and an emission maximum of around 525 nm. Fluorescence of the reagent increases by approximately 1,000-fold when bound to RNA. A serial dilution of RNA samples is prepared and mixed with the reagent. The emission fluorescence is measured and a linear calibration curve of template concentration and emitted fluorescence intensity is used to measure the concentration of sample RNA.
! CAUTION Calculating concentrations from absorbance readings is not sufficiently accurate for use with RT-qPCR technology[51]. Select one method for RNA quantification and use this consistently. Do not attempt to compare data from different procedures, as all commonly used methods generate different results[12].

(A) Nanodrop ● TIMING 2 min per sample

After blanking and setting the system to zero with 1 µl of distilled water, place 1 µl of RNA onto the sensor and measure the RNA concentration; the instrument automatically calculates the RNA concentration. The NanoDrop ND-1000 Spectrophotometer (260/280 nm) can measure 1 µl samples with concentrations between 2 ng/µl and 3,000 ng/µl without dilution.

▲ CRITICAL STEP The sensor must be wiped clean between samples, and once quantification is complete, wash the sensor using 1 µl distilled water.

(B) RiboGreen RNA quantification ● TIMING 1 h

(i) Make up a master mix of 1:200 RiboGreen in TE.

(ii) Prepare a dilution series of the rRNA standard (provided in the RiboGreen kit at 100 ng/µl) covering the expected concentration range of the RNA. After an initial dilution of 1:100, prepare a two-fold serial dilution series starting at 1 ng/µl (see table below). All dilutions are carried out in TE.

Relative RNA concentrations in RiboGreen assay standards.

Dilution	Input RNA concentration
1:1	1.0 ng/µl
1:2	0.5 ng/µl
1:4	0.25 ng/µl
1:8	0.125 ng/µl
1:16	0.0625 ng/µl
1:32	0.0313 ng/µl
1:64	0.0156 ng/µl
1:128	0.0078 ng/µl

(iii) Add an equal volume of the 1:200 RiboGreen mastermix to each standard dilution and to the RNA sample to be quantified.

(iv) Use a real-time PCR instrument or fluorimeter to quantitate nucleic acids in the detection range. RiboGreen-based assays are significantly more sensitive than A260 measurements and have a linear dynamic range extending from 1 ng/ml to 1 µg/ml RNA using two different dye concentrations. Analyze each standard in triplicate for greatest reliability. This requires at least 45 µl of each standard; so a total of 50 µl of each serial dilution is suggested. Note that, once the RiboGreen dye has been added, the actual concentrations of RNA in the assay will be half of those in the table above.

(v) Using the readouts for each standard from the step above, plot a standard curve of fluorescent emission against input rRNA concentration.

(vi) Compare the readout for the RNA sample to the standard curve to determine its concentration.

Analysis of total RNA integrity ● TIMING 30 min per chip

11| The conventional approach to determining RNA integrity uses agarose gel electrophoresis[52]. Although conventional agarose gel electrophoresis can be used to visualize RNA, the addition of formaldehyde denatures the high secondary structure of the RNA molecule. However, since this uses relatively large amounts of RNA, it is better to use either the Agilent 2100 Bioanalyzer or BioRad Experion to provide a quality assessment of rRNA based on comparison of peak areas to the combined area of the six reference RNAs. This will also provide an alternative estimate of RNA quantity. Carefully follow the instructions provided with

the LabChips. Heat RNA samples and the RNA ladder to 70 °C for 2 min before use and then keep on ice and load within approximately 10 min.

▲ CRITICAL STEP Poor LabChip loading and formation of salt bridges between electrodes are common causes of poor assay performance. Overloading the chip with RNA (more than 500 ng) generates unreliable results. The sample's ionic strength affects the accuracy of quantification, as it can quench fluorescence. The RNA loaded onto the LabChip should be diluted in RNase-free water, as otherwise its concentration may be underestimated. Since only 1 µl of sample is loaded, accurate pipetting is crucial for obtaining accurate quantification. Quantification will also be inaccurate if the entire sample does not enter the gel, something that is difficult to ascertain. Consequently we prefer to quantitate separately as described above.

Analysis of mRNA integrity ● TIMING 5 h

12| Make an RT mastermix in 1.5 or 2 ml microfuge tubes by mixing the reaction components in the order stated in the table below.

▲ CRITICAL STEP Prepare no-RT controls in duplicate: to prepare each no-RT control add 18.75 µl mastermix to 6.25 µl water, prior to adding RT enzyme to the mastermix.

mRNA integrity assay RT mastermix components.

Component	Final concentration	Volume per reaction (µl)
Water		12.75
10× RT buffer	1×	2.5
Oligo-dT primer (500 ng/µl)	50 ng/µl	2.5
dNTP (100 mM)	4 mM	1
Reverse transcriptase 50 U/µl	2.5 U/µl	1.25

13| Add 20 µl mastermix to 10–100 ng of each RNA sample in a volume of 5 µl. Ensure that the final reaction volume is 25 µl.

14| Incubate at 20 °C for 10 min followed by 50 °C for 60 min.

15| Terminate the reaction by heating to 85 °C for 5 min, and then place on ice for 2 min or until required. Collect by brief centrifugation.

■ PAUSE POINT This cDNA can be stored at –20 °C for up to 6 months.

16| Make a qPCR mastermix by adding the reagents in the order shown in the table below. Alternatively, a 2× commercially available qPCR buffer can be used with a final $MgCl_2$ concentration of 4 mM. Note that the set volumes assume addition of reference dye ROX and cDNA templates in volumes of 0.375 µl/reaction or 5 µl/reaction, respectively. Where conditions require a deviation from this protocol adjust water volume accordingly to result in a final reaction volume of 25 µl.

mRNA integrity assay qPCR mastermix components.

Master mix	Final concentration	1× reaction (µl)
dH_2O		8.525
Buffer 10×[a]	1×	2.5
50 mM $MgCl_2$	4 mM	2.5
dNTP 20 mM	0.8 mM	1
10 µM each of six primers (forward and reverse for 5′, center and 3′ GAPDH primers, **Table 1**)	240 nM each primer	3.6 [0.6 µl of each of six primers]
1 mM ROX[b] (dilute to give desired final concentration)	30 or 300 nM	0.375
Probes 5 µM (5′, center and 3′ GAPDH probes, **Table 1**)	100 nM each	1.5 [0.5 µl each of three probes]

[a]Commercial mastermixes are available in 2× concentration. These are also suitable but note Mg^{2+} concentration and adjust accordingly. [b]Addition of ROX as a passive reference dye is optional. Its fluorescence does not change during the course of the PCR reaction but provides a stable baseline to which samples are normalized; this compensates for changes in fluorescence between wells caused by slight volume differences in reaction tubes and due to optical difference in the measuring systems of the instruments. For tungsten/halogen lamp-based instruments and instruments equipped with a ~584 nm LED use the reference dye at a final concentration of 30 nM. If using laser-based instruments (ABI PRISM 7700 and 7900HT), use the reference dye at a final concentration of 300 nM.

▲ CRITICAL STEP When using a reference dye prepare fresh dilutions of ROX (or dye provided) using nuclease-free PCR-grade water prior to setting up the reactions, and keep all tubes protected from light as much as possible. Throw away any remaining diluted ROX at the end of the day. Invitrogen is a source of reliable ready-to-use ROX (cat. no. 12223-012).

17| Standard curve template: Prepare five 10-fold serial dilutions into yeast tRNA (100 ng/µl) of target-specific amplicon using a range of approximately 10^5 to 10 copies. Alternatively, use six 5-fold dilutions of cDNA prepared from universal RNA (Stratagene cat. no. 750500 (human), 7410100 (mouse), 740200 (rat)). In each case, carry out the reactions in duplicate.

18| Add 5 µl standard curve template to the appropriate qPCR reaction tubes or wells.

19| Perform a 1:10 dilution of the cDNA from Step 15 and add 5 μl of each sample to two qPCR reaction tubes.

20| Add 20 μl master mix to individual reaction tubes, mix gently by repeatedly pipetting up and down, avoiding bubbles. Briefly spin reactions to the base of the reaction tube.

21| Run in qPCR instrument using a three-step protocol. The precise cycling conditions depend on the type of enzyme and instrument used. The following conditions are universally accepted for conventional block systems and are also appropriate for Corbett Rotorgene instruments:

1 cycle	Activation	95 °C 10 min
45 cycles	Denaturation	95 °C 30 s
	Annealing	56 °C 30 s
	Extension	62 °C 30 s (collect data during each cycle)

22| Check the baseline and threshold settings, and analyze the plot of log template concentration with respect to C_t as described in the data analysis section (Steps 45–58).

23| Analyze each assay individually (i.e. 5′, center or 3′) by referring to the standard curve generated in Step 22 to quantify the number of copies of each target; the standard curve controls for differences in reaction efficiencies and ensures a more accurate quantification.

24| Calculate the ratio of each target. For example, if the 5′ amplicon has a calculated copy number of 1.46×10^7 and the 3′ amplicon has a calculated copy number of 1.78×10^7, then the 3′:5′ ratio of that sample is $1.78 \times 10^7/1.46 \times 10^7$, or 1.22 and is indicative of good quality RNA. On the other hand, if the relative copy numbers are 1.18×10^5 (5′) and 1.78×10^7 (3′), then the resulting ratio of 151 suggests that the mRNA is degraded. While it may still be acceptable to use such RNA in an RT-qPCR assay, especially when using specific primers, it is best not to directly compare results obtained from such RNA with results obtained from high quality RNA. As a general guideline consider RNA with a 3′:5′ ratio of <5 to be high quality and suitable for any downstream applications.

Assay to determine presence of RT-qPCR inhibitors ● TIMING 3 h

25| Add components in the order stated in the table below to prepare a qPCR mastermix sufficient for all RNA samples to be tested in duplicate. Note that the given protocol includes the use of a reference dye (ROX); if its use is not required, adjust water volume accordingly.

▲ **CRITICAL STEP** Include a minimum of two 'no sample' controls (i.e., where the only template is SPUD-A).

SPUD inhibitor detection assay RT-qPCR mastermix components.

	Stock concentration	Volume to add for single 25 μl reaction
Water	Assuming template is in 3 μl	2.425 μl
Mastermix (qPCR)	2×	12.5 μl
$MgCl_2$ (25 mM)	3 mM	3 μl
Reference dye (ROX)[a]	0.03 μM (optional depending on instrument)	0.375 μl
Amplicon (SPUD-A)	20 pmol/μl	2 μl
Probe (SPUD P)	5 pmol/μl	0.5 μl
Primers (SPUD F and R mixed)	10 pmol/μl each	1.2 μl

[a]Note that the requirement of the reference dye is instrument-dependent. Alternatively follow a two-tube RT-qPCR protocol and then add 0.5 μl of 1/10 diluted cDNA to qPCR mastermix.

26| Add 3 μl of each sample RNA template (at around 25 ng/μl) to two qPCR tubes. Add 22 μl mastermix to each RNA sample.

27| Run in qPCR instrument using the two-step protocol outlined in the table below:

1 cycle	Activation	95 °C 10 min
40 cycles[a]	Denaturation	95 °C 30 s
	Annealing/extension	62 °C 60 s (collect data during each cycle)

[a]The precise cycling conditions depend on the type of enzyme and instrument used.

28| Determine the C_t value for the control reaction containing SPUD-A alone (i.e., no sample RNA), referring to the data analysis section (Steps 45–50) for general guidelines.

29| Using exactly the same threshold setting, determine C_t for reactions containing test samples and compare to the control. The presence of inhibitors is indicated by a higher C_t being recorded for test samples than for the control containing SPUD-A

TABLE 2 | Forward and reverse primer concentrations for optimization assay.

Primer stock (F) → (R)↓	0.5 μM	1 μM	2 μM	3 μM
0.5 μM	50/50	100/50	200/50	300/50
1 μM	50/100	100/100	200/100	300/100
2 μM	50/200	100/200	200/200	300/200
3 μM	50/300	100/300	200/300	300/300

Primer concentrations are stated as forward primer/reverse primer in nM in the final qPCR reaction.

alone. The distribution of the C_t values from the duplicate samples containing SPUD A alone indicates its coefficient of variance (CV) and defines the acceptable range of C_t values for samples which do not contain inhibitors. All samples showing a shift in C_t greater than these values should be purified or a fresh RNA sample extracted. ▲ CRITICAL STEP Do not adjust threshold settings when comparing C_t of controls and samples.

PCR Optimization ● TIMING 4 h

30| The target sample used for optimization can be an artificial amplicon (best for reliable quantification), linearized plasmid, PCR product or cDNA. When using an artificial amplicon or linearized plasmid as the template to determine the PCR efficiency, prepare a stock of target sample of around 10^8 copies amplicon/μl. Dilute the selected stock template through five ten-fold serial dilutions. If cDNA synthesized from universal RNA is to be used, 0.5–1 μl is usually sufficient to detect even low abundant messages. Dilute the selected stock template through five five-fold serial dilutions.

31| Test the amplification of the template using 100–200 nM of each primer. Choose the template concentration for which amplification results in a C_t of between 20 and 30. If the concentration is outside of this range assume that a 10-fold difference in concentration corresponds to 3.3 cycles and select the appropriate samples to use accordingly.

32| Prepare individual working dilutions of forward and reverse primers for preparing an optimization matrix (see **Table 2**). Prepare 20 μl each of 0.5 μM, 1 μM and 3 μM primer stocks.

33| Prepare a matrix of primer concentrations by adding 2.5 μl of primer at the appropriate stock concentration to two qPCR reaction tubes, such that each reaction is prepared in duplicate. Refer to **Table 2** for the final concentrations of primers.

34| Prepare a qPCR mastermix according to the table below.
▲ CRITICAL STEP Before adding sample to the mix, remember to create two NTC (no template control) reactions using minimum and maximum primer concentrations.

qPCR mastermix for primer optimization assay.

	Stock concentration	Volume to add for single 25 μl reaction
Water		2.125 μl
SYBR Green I Mastermix (qPCR)	2×	12.5 μl
Reference dye (ROX)[a]	0.03 μM (optional depending on instrument)	0.375 μl
Sample	10^4 to 10^5 copies or 5 μl 1:10 diluted cDNA synthesis reaction or alternative template from Steps 30,31)	5 μl

[a]Note that the given protocol includes the use of the ROX reference dye; if this is not required, adjust water volume accordingly.

35| Add 20 μl mastermix to each primer pair in qPCR tubes. Mix, avoiding bubbles, and briefly spin reaction tubes to ensure components are in the bottom of tubes.

36| Run in qPCR instrument using the following three-step protocol:

1 cycle	Activation	95 °C for 10 min
40 cycles	Denaturation	95 °C for 30 s
	Annealing	60 °C for 30 s (collect data)
	Extension	72 °C for 30 s
1 cycle	Melt curve[1]	Between 55 °C and 95 °C (collect data at each temperature)

[1]The melt curve profiles are instrument-dependent; default settings are suitable to enable visualization of primer dimers or non-specific amplification.

37| Referring to Steps 45–50 for general guidelines for data analysis, look at melt curves and amplification plots and select the primer combination that fulfils the following criteria in the given order of priority: (i) absence of, or minimal, primer

dimers; (ii) lowest C_t i.e. the primer combination resulting in the most sensitive and efficient reaction; (iii) highest end point fluorescence (ΔRn), which is likely to signify the highest number of amplicon products being formed; and (iv) absence of signal in the NTC.

Determination of assay sensitivity and reproducibility ● TIMING 3 h

38| Prepare a 10-fold serial dilution of the template described in Step 30 in 100 ng/μl yeast tRNA using a minimum of five samples covering the range between 10^7 and 10 copies in a volume of 5 μl. Although it is possible to quantify a dynamic range of 10 or 11 logs on most systems (i.e., from 1 to 10^{10} or 10^{11} target copies), in practice this is not really necessary.
▲ CRITICAL STEP Ensure that the dynamic range spans the C_t values anticipated for experimental analysis, repeating the dilution series using different concentrations if required (**Fig. 5**). It is not valid to extrapolate quantification values of test samples beyond the values of the samples in the dilution series tested. It is remarkable how many examples there are of copy numbers calculated for unknown samples from extrapolated standard curves. This is unacceptable since the conditions for quantification of standard curve samples are invariably more favorable than those for the test samples.

39| Add 5 μl of each dilution point to qPCR reaction tubes, in duplicate.

40| Prepare a qPCR mastermix according to the table below, using the primer concentrations determined in Steps 36 and 37 from the primer concentration optimization process.

qPCR mastermix for determination of assay efficiency and sensitivity.

	Stock concentration	Volume to add for single 25 μl reaction
Water		2.125 μl
SYBR Green I Mastermix (qPCR)	2×	12.5 μl
Reference dye (ROX)[a]	0.03 μM (optional depending on instrument)	0.375 μl
Primers	As determined during primer optimization process	5 μl

[a]Note that the given protocol includes the use of the ROX reference dye.

▲ CRITICAL STEP Quality assess all new batches of primers or probes. There can be a wide variation between batches, even when sourced from the same supplier. Always verify primer concentrations by testing on a serial dilution of template as described in the above protocol.

41| Add 20 μl mastermix to each sample in qPCR tubes. Mix, avoiding bubbles.

42| Run in qPCR instrument using three-step protocol below:

1 cycle	Activation	95 °C 10 min
40 cycles	Denaturation	95 °C 30 s
	Annealing/	60 °C 30 s (collect data during each cycle)
	Extension	72 °C 30 s
1 cycle	Melt curve[a]	Between 55 °C and 95 °C (collect data at each temperature)

[a]The melt curve profiles are instrument-dependent, default settings are suitable to enable visualization of primer dimers or nonspecific amplification.

43| Following the steps outlined in the data analysis section (Steps 45–58 correct), use the instrument software to plot C_t against log target concentration and determine the slope and R^2 values. Confirm that the slope lies between −3.2 and −3.5 and that the R^2 value is above 0.98, as described for standard curve analysis (Step 45–58 correct).
▲ CRITICAL STEP Perform the qPCR as soon as possible after preparing the reaction mixes. Some assays are stable for 12 h if stored at 4 °C overnight; however others are more sensitive to storage. Reactions containing glycerol and DMSO or made from commercial master mixes can be stored at −20 °C for up to 12 h.

RT-qPCR assay

44| The RT-qPCR assay can be carried out as a two-tube reaction (option A) or as a single-tube reaction (option B).

(A) Two-tube RT-qPCR protocol ● TIMING 4–4.5 h

(i) The RT reaction can be carried out using random priming, oligo-dT priming or gene-specific priming. See flow chart for a summary of the steps (**Fig. 6**) and **Box 3** for advice on minimizing variation between RT reactions. Mix and briefly spin RNA and primers and combine the following in a microfuge tube:
▲ CRITICAL STEP Keep reagents and microfuge tubes on ice when setting up the reaction described below.

RNA/primer mix components.

Component	Volume per reaction		
	Random	Oligo-dT	Gene-specific
Total RNA 1–200 ng (max. 500 ng)	(max 7 μl)	(max 7 μl)	(max 7 μl)
Random 15-mer (50 ng/μl)	1μl	_	_
Oligo-dT (500 ng/μl)	_	1 μl	_
3′ (antisense) primer (2 μM)	_	_	1 μl
Water	to 10 μl	to 10 μl	to 10 μl

(ii) Incubate at 65 °C for 10 min, then snap cool on ice for 5 min.

(iii) Prepare a reverse transcription mastermix by adding each of the components listed on the RT mastermix components table below to a microfuge tube.

RT mastermix components.

Component	Volume per reaction
10× RT buffer	2.5 μl
$MgCl_2$ (25 μM)	5 μl
DTT (0.1 M)	2.5 μl
RT enzyme (200 U/μl)	1 μl
Water	to 15 μl

(iv) Add 15 μl of the RT mastermix to the RNA/primer mix. Gently mix the tube contents and briefly spin.
▲ **CRITICAL STEP** Prepare no-RT controls in duplicate. To prepare each no-RT control, add 18 μl mastermix to 7 μl water prior to adding RT enzyme to the mastermix.

(v) For random- or oligo-dT priming, incubate at 20 °C for 10 min followed by 50 °C for 60 min. For gene-specific priming, incubate at 60 °C for 15 min.

(vi) Terminate the reaction at 85 °C for 5 min, and then place on ice for 5 min. Collect by brief centrifugation.
■ **PAUSE POINT** First strand synthesis can be stored at −20 °C for at least 6 months.

(vii) qPCR reaction: qPCR can be carried out using either "non-specific" (e.g., SYBR Green I dye) or probe-based (e.g., TaqMan) chemistries. Make a master mix by adding the reagents in the order shown, according to the appropriate column in the table below. Mix gently by repeatedly pipetting up and down (making sure there are no bubbles), and finally add Taq polymerase and mix gently again.

qPCR mastermix components.

Master mix	Final concentration	Volume per reaction (μl)	
		SYBR Green I dye[a]	TaqMan[a]
dH_2O		14.125	12.125
10× buffer	1×	2.5	2.5
50 mM $MgCl_2$	2.5 mM (5 mM for TaqMan)	1.25	2.5
20 mM dNTP	0.8 mM	1	1
10 μM Primers	Refer to optimization results	0.25	0.25
ROX diluted (optional)	Refer to instrument manufacturer's recommendations	0.375	0.375
SYBR Green I diluted 1:50,000		0.25	_
5 μM probe	200 nM	_	1
5 U/μl Taq		0.25	0.25

[a]Buffer, $MgCl_2$, dNTPs, *Taq* and (for non-specific assays only) SYBR Green I dye are combined in commercial 2x mastermix preparations. ROX is also included in some cases. In addition these contain enzyme stabilizing reagents. Note that this protocol includes the use of the reference dye ROX; if this is not required, adjust water volume accordingly.

(viii) Add 5 μl template to the reaction tubes or wells.

(ix) Add 20 μl of reaction mix to each tube or well containing template. Cap carefully, spin briefly to ensure there are no bubbles, and place the tubes, strips, rotor or 96/384-well plate into the real time thermal cycler.

(x) For SYBR Green I dye assays, perform a three-step PCR reaction according to the thermal profile below:

Three-step qPCR cycling conditions for SYBR Green I assays.

1 cycle	Activation	95 °C	10 min	
40 cycles	Denaturation	95 °C	15 s	
	Annealing[a]	60 °C	30 s	Collect data
	Extension	72 °C	30 s	Collect data

[a]Annealing temperature is primer design dependent (see REAGENT SETUP).

For dual-labeled probe assays, perform a two-step PCR reaction according to the thermal profile outlined below.

Two-step qPCR cycling conditions for dual-labeled probe assays.

1 cycle	Activation	95 °C	10 min	
40 cycles	Denaturation	95 °C	15 s	
	Annealing/extension	62 °C	1 min	Collect data

Note that reactions containing hydrolysis probes are usually performed using a two-step PCR profile. During this reaction the double-stranded template is melted at 95 °C and the annealing and extension steps both occur during a single incubation at 62 °C. Although this is suboptimal for amplification by *Taq* polymerase it is believed to encourage more efficient cleavage of the internal probe, resulting in maximum fluorescent signal per cycle. For optimal amplification and signal detection, we recommended a three-step protocol for experiments including other probe-based chemistries (e.g., Molecular Beacons, Scorpions).

For SYBR Green I dye assays, obtain a melting profile (as below or according to the instrument manufacturer's instructions, see INTRODUCTION and REAGENT SETUP).

1 cycle	95 °C	1 min	
41 cycles	55 °C	30 s	Collect data
	Repeat and increase temperature by 1 °C per cycle		Collect data

(B) One-tube RT-qPCR protocol ● TIMING 3 h

(i) One tube RT-qPCR can be carried out using either "non-specific" (e.g., SYBR Green I) or probe-based (e.g., TaqMan) chemistries. Make a master mix by adding the reagents in the order shown in the table below, according to the appropriate column. Mix gently by repeatedly pipetting up and down (making sure there are no bubbles), and finally add *Taq* polymerase and mix gently again. Note that buffer, $MgCl_2$, dNTPs, SYBR Green I dye and *Taq* are combined in commercial 2x mastermix preparations.

RT-qPCR mastermix components.

Master mix	Final concentration	Volume per reaction (µl)	
		SYBR Green I dye[a]	Probe based detection
dH_2O		5.25	4.25
2× SYBR Green I RT-qPCR mastermix buffer[1]	1×	12.5	_
2× RT-qPCR mastermix buffer[1]	1×	_	12.5
10 µM Primers	Refer to optimization results	0.25	0.25
5 µM Probe	200 nM	_	1.0
ROX diluted (optional)	Refer to instrument manufacturer's recommendations	0.5	0.5
Diluted RT enzyme	Refer to reagent manufacturer's recommendations	1.5	1.5

[a]Use one of the commercial one tube RT-qPCR buffers. Note specific instructions from the manufacturer. This protocol includes the use of the reference dye ROX; if this is not required, adjust water volume accordingly.

(ii) Add 5 µl template to the reaction tubes or wells.

(iii) Add 20 µl of reaction mix to each tube or well containing template. Cap carefully, spin briefly to ensure there are no bubbles, and place the tubes, strips, rotor or 96/384-well plate into the real time thermal cycler.
For SYBR Green I dye assays, perform an RT-qPCR reaction according to the thermal profile below:

One-tube RT-qPCR cycling conditions for SYBR Green I assays[a].

1 cycle	First strand synthesis	50 °C[a]	15 min	
	Denaturation/ Activation	95 °C	30 s	
1 cycle	Activation	95 °C	10 min	
40 cycles	Denaturation	95 °C	15 s	
	Annealing	60 °C	30 s	Collect data
	Extension	72 °C	30 s	Collect data

[a]The RT incubation step should be determined experimentally and can range between 45 °C and 60 °C. This is template- and enzyme-specific. Annealing temperature is primer design-dependent (see REAGENT SETUP).

For probe-based assays, perform an RT-qPCR reaction according to the thermal profile below:

One-tube RT-qPCR cycling conditions for dual-labeled probe assays.

1 cycle	First strand synthesis	50 °C[a]	15 min	
	Denaturation/ Activation	95 °C	30 s	
1 cycle	Activation	95 °C	10 min	
40 cycles	Denaturation	95 °C	15 s	
	Annealing	62 °C	30 s	Collect data

[a]The RT incubation step should be determined experimentally and can range between 45 °C and 60 °C. This is template and enzyme specific. Annealing temperature is primer design dependent (see REAGENT SETUP).

For SYBR Green I assays, obtain a melting profile (as below or according to the instrument manufacturer's instructions, see INTRODUCTION and REAGENT SETUP).

1 cycle	95 °C	1 min	
41 cycles	55 °C	30 s	Collect data
	Repeat and increase temperature by 1 °C per cycle		Collect data

Data analysis ● **TIMING 2.5 h**

! CAUTION The analysis of qPCR data can be highly subjective, since C_t values can be altered by changes to baselines and threshold levels. Although software default options can assuage some subjectivity, it is also true that these settings are not always appropriate. Clearly, it is important to understand the analysis procedure in order to be able to generate reliable data. The steps below have been designed to enable standardization of analysis techniques and the criteria with which data should be accepted and reported.

45| Examine amplification plots of standard curves or reference samples and identify abnormal plots. A normal amplification plot is defined as consisting of a linear baseline region, a log phase of amplification, followed by a plateau (**Fig. 7**).

46| Make a note of samples that may record a C_t, but where the amplification plot clearly does not show the appropriate characteristics (**Fig. 8**, purple, yellow and blue curves). These must be subjected to further investigation and either corrected by following the steps outlined below or discarded. When a software solution is not identified the reaction should be repeated.

47| Check background fluorescence levels (high background levels result in lower sensitivity). Also check NTC background fluorescence levels and ensure that there is not a significant upwards drift. A subtle increase can indicate that the probe is degrading during cycling.

48| Check baseline settings and that all the data constituting the baseline are set at zero. It is usual for the data from the initial cycles to show an apparent decrease or increase in fluorescence. These data are excluded from the baseline by instructing the software to start at the cycle where the horizontal region begins. In the case of the data in **Figure 9a** where the plots are seen to

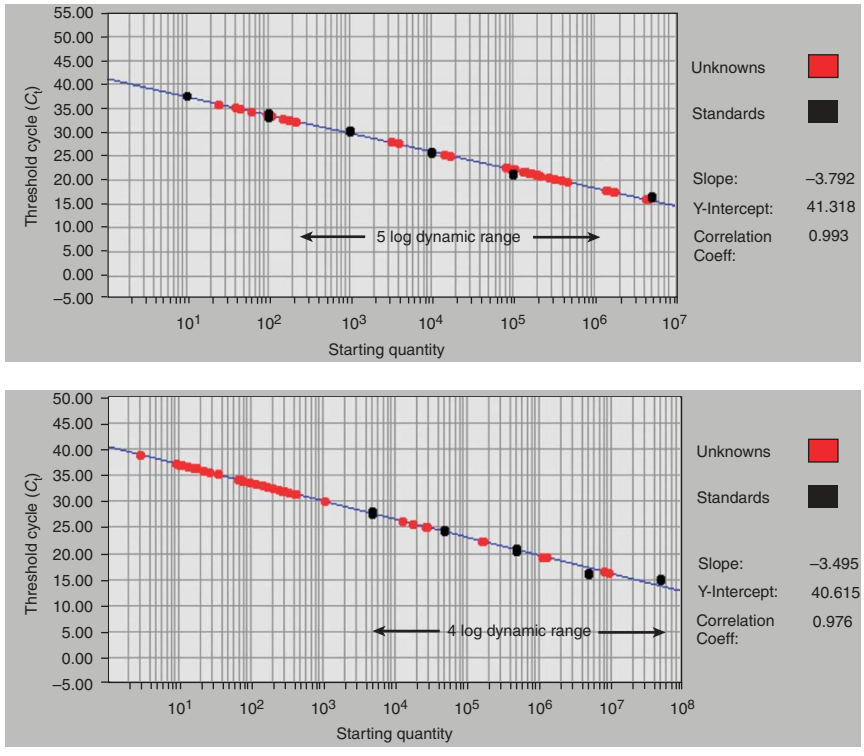

Figure 5 | Acceptable and unacceptable standard curves. All the unknowns (red dots) in the upper picture are contained within the dynamic range of the standard curve, which is demarcated by the two outermost points of the standard represented by black dots. This allows accurate quantification of the corresponding mRNAs. In the lower picture, a majority of the unknowns is below the highest dilution of the standards (4×10^3 copies).

decrease between cycles 1 and 25, the software needs to be instructed to interpret the curves such that it recognized cycles 1 to 25 as the baseline region. After this procedure has been carried out the baseline region becomes horizontal and zero (see corrected data in **Fig. 9b**). It is appropriate to include a different number of cycles in the baseline for different samples. For samples with a low C_t, fewer cycles will be included with the baseline compared with samples recording a higher C_t.

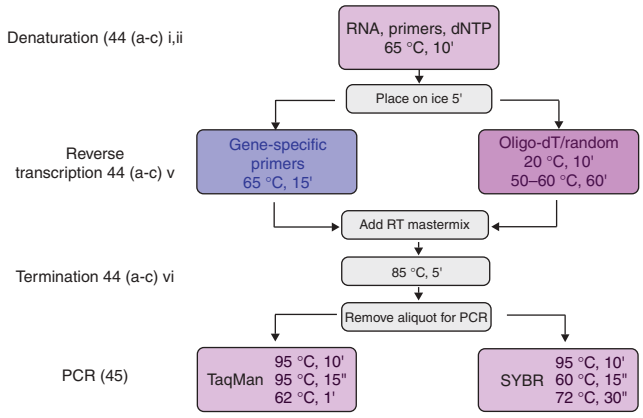

Figure 6 | Outline of reverse transcription protocol. The detailed procedures are described in Steps 44 and 45.

49| After software adjustments, remove all remaining abnormal plots (those identified in Step 46) from the analysis.

50| The threshold defines the fluorescent reading at which all measurements are taken and setting the threshold is also vulnerable to subjectivity. View data on a log linear scale. Set the threshold level in the log region of amplification, where all amplification plots are parallel and above the background noise of the baseline. If the amplification plots are not parallel quantification will be inaccurate. ▲ **CRITICAL STEP** Do not move threshold settings when comparing samples to each other, a reference sample or standard curve. When taking readings for data that are to be analyzed relative to each other, it is critical that the threshold setting remains constant, for example measuring the same target in unknown samples with respect to the relevant standard curve or reference sample. It is appropriate to adjust the threshold to take readings for a different target in the same or different samples and with the same standard curve samples. This is particularly relevant when the reactions are run in multiplex and a different dye has been used.

51| Check slope of the standard curve is between −3.2 and −3.5 and reproducibility of replicates $R^2 > 0.98$.

52| Check controls for expected results.

53| Check melt curve profiles for fluorescent nucleic acid binding dye detection and ensure that products are specific. **Figure 10** shows a melt curve with two peaks, the one at the higher temperature caused by the amplification of genomic DNA, which contains a short intron. The melt curves in **Figure 11** show the effects of varying input template concentrations, with increasing amounts of primer dimers becoming apparent at low input RNA concentration.

54| Where possible, ensure that the C_t of the positive control (or dilutions of standard curve) is within 1 C_t of that recorded during a previous experiment and that the initial and final fluorescence levels are similar.

55| Examine amplification plots for samples to be measured as described for the standard curve or reference sample.

56| Ensure that all data points to be recorded are within the dynamic range defined by the standard curve.

57| Examine replicates. All replicates should be within 0.5 C_t of each other. At low C_t the tolerance should be lower than at high C_t. Above cycle 35 the variability will be greater and quantification may be unreliable.

58| Ensure a minimum difference of 5 C_t between any NTC signal and a sample data point[4]. When using fluorescent nucleic acid binding dyes take care with C_t values when primer dimer products are evident in the sample after melt curve analysis.

Normalization ● **TIMING 30 min**

59| All RT-qPCR assay results are subject to variability caused by technical as well as biological variation. It is essential that technical variability is kept at a minimum so as to optimize the chances of identifying biologically relevant changes in mRNA

BOX 3 | VARIATION BETWEEN RT EXPERIMENTS

When using random primers, the efficiency of the RT reaction may vary between reactions. It also appears that the RT efficiency for different targets within the same reaction is variable[38] and it is important to control for these factors by following the steps below:

- When possible, perform RT reactions on all samples at the same time.
- Use the same amount of RNA in all reactions.
- When multiple samples are processed in different batches, include a common positive reference sample alongside all batches.
- Quantify target genes relative to a standard curve including the target copy number in the positive control samples from each batch.
- Express quantity of target in samples relative to the quantity measured in the control sample included in the same RT batch[38].

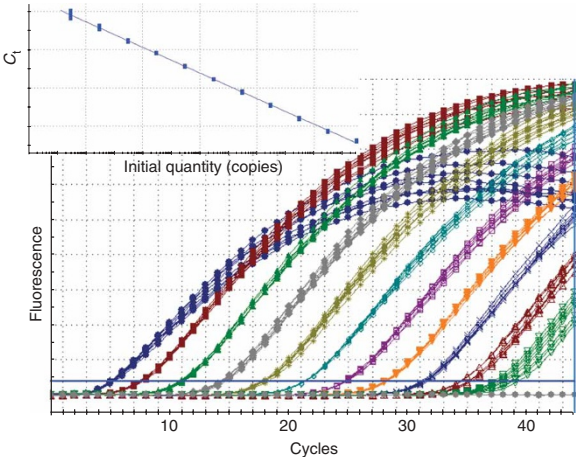

Figure 7 | The relationship between amplification plot and standard curve. The amplification plots were generated by serial ten-fold dilutions of an RNA sample containing 3×10^{11} targets. Eight replicates were run for each dilution point. The horizontal blue line marks the threshold, at which the C_t values are obtained. The standard curve (insert) generated from these amplification plots is linear over 11 logs, with a slope of –3.323 and an R^2 of 0.999.

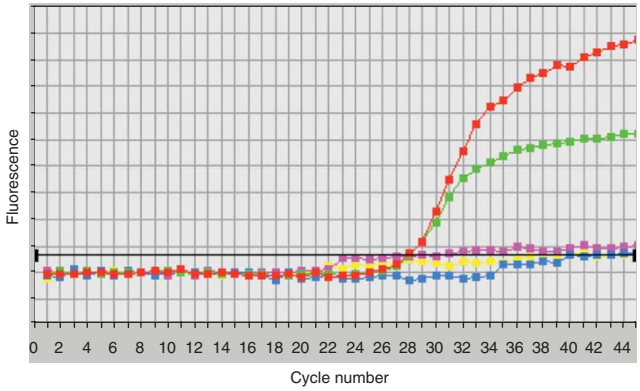

Figure 8 | Invalid C_t generated by spurious amplification plot. The green and red amplification plots are from two replicates that record identical C_ts, albeit with different ΔR_n values, and are typical examples of genuine amplification. The remaining three samples (purple, yellow and blue) record positive C_ts, but these are clearly not due to genuine target amplification. The horizontal black line indicates the threshold. Reliance on the experimental report generated automatically by the real-time instrument's software would result in the reporting of a false positive result.

levels. Consequently, data normalization is an essential part of a meaningful RT-qPCR assay and requires standardization. Unfortunately, normalization is a rather problematic area and, as yet, there is no universally accepted method for data normalization that accounts for all variables encountered during the course of a RT-qPCR experiment. All proposed methods represent a compromise and the selection depends upon the study aims and acceptable tolerance[53]. Since a detailed discussion of normalization is beyond the scope of this protocol, we present three options for data normalization: (A) input RNA amount; (B) endogenous reference genes; and (C) genomic DNA. It is also possible to normalize against area dissected when using LCM, or cell number when extracting RNA from nucleated blood cells, but these are not discussed below.

(A) Normalization against input RNA amount

This is feasible and, if validated, perfectly acceptable[7]. However, there are problems comparing tissues that are proliferating at different rates, since they will contain different amounts of rRNA and the mRNA/rRNA ratios are likely to be different. Furthermore, differences in cDNA synthesis are not taken into account and in some situations (e.g., embryology) may generate irrelevant results.

(B) Normalizing to an endogenous reference (not "housekeeping") gene or genes

This is the most common method for internally controlling for error in RT-qPCR

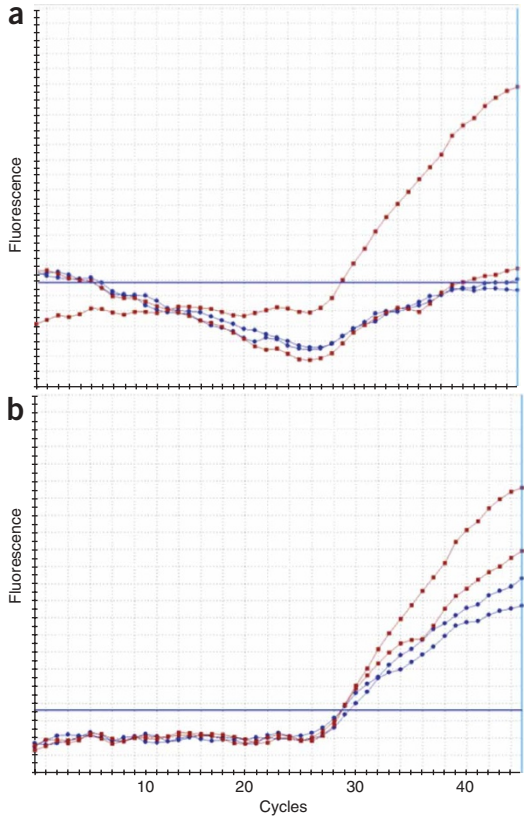

Figure 9 | Amplification plots demonstrating the importance of appropriately adjusting the baseline. Two replicates of RNA extracted from a colorectal cancer sample were amplified using IGF-I-specific primers. (**a**) The amplification plots obtained using an unadjusted baseline (calculated from cycles 5–15) shows an apparent problem with the second replicate from the first reaction (red), as well as poor amplification of the second set of replicate samples (blue). However, a visual inspection of the plots suggests a downward drift in the baseline. (**b**) The use of cycles 5–26 to recalculate the baseline results in identical C_ts for the replicates in sample 1 and near-identical C_ts for the replicates in sample 2. The threshold is represented by the horizontal blue line.

and is currently the preferred option, as long as it is carried out correctly[54]. The inevitable consequence of inappropriate normalization is the acquisition of biologically irrelevant data[55,56]. This strategy expresses target gene levels relative to those of internal reference genes and benefits from the fact that all the steps required to obtain the final PCR measurement are controlled for. The procedure is simplified as both the gene of interest and the reference gene are measured using real-time RT-PCR. However, it is essential that reference gene expression in the target tissue is carefully analyzed[57] and the minimum variability is determined and reported[58]. The current gold standard combines the evaluation of a panel of several reference genes (e.g., Bioline cat. no. 35030) together with a method for selecting reference genes with the most stable expression (e.g., GeNorm[58] medgen.ugent.be/~jvdesomp/genorm/ or Bestkeeper[59]).

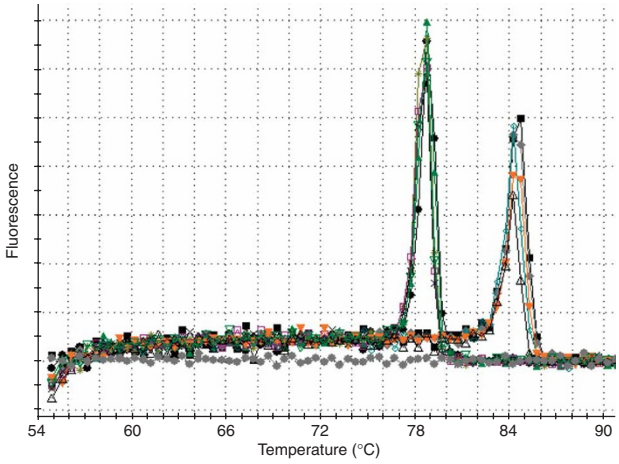

Figure 10 | SYBR Green melt curve demonstrating the co-amplification of an mRNA target (lower T_m) and genomic DNA with a short intron. The identity of the peaks can be determined from the T_m without the need for sequencing.

(C) Normalization against gDNA

Since mRNAs are copied from genomic DNA, another way to express mRNA level is as copy number per template DNA, or more practically, as copy number per cell[60]. This is particularly useful when performing RT-qPCR analyses on RNA obtained from minute samples using kits such as the Invitrogen CellsDirect kit (see above). The main problems here are that (i) differential stability of DNA and RNA may distort quantification, (ii) sample cannot be DNase-treated and (iii) whilst being an internal control, there is no equivalent RT step.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Problem	Possible reason	Solution
General		
Normalization of RNA samples based on A_{260} measurement results in samples of unequal concentration.	RNA may be degraded – absorbance increases in the presence of free nucleotides, leading to overestimation RNA concentration.	Check RNA sample using automated or agarose gel electrophoresis.
Determination of RNA concentration by RiboGreen is unreliable.	False readings can be obtained if RiboGreen absorbs to the sides of the tube.	Use non-stick, nuclease-free polypropylene tubes for RiboGreen assays.
No amplification detected.	No amplification occurring.	Resolve PCR products by agarose gel electrophoresis.
	Probe-based detection failing.	Try fluorescent nucleic acid binding dye detection.
Amplification plot increases very little when using probes for detection.	High background – instrument problem.	Probe inefficiently quenched.
	Low background.	Probe poorly labeled; instrument not detecting dye; Probe has G as 5′ base; Probe is designed to overlap primer site; probe is too far from 3′ of primer and so is being displaced rather than cleaved.
Amplification plot dips under baseline.	Baseline setting is using an incorrect end cycle.	Examine raw data and reset baseline cycles.
Replicates show high variability.	Poor assay efficiency. Poor pipetting.	Redesign assay.
C_t recorded in NTC.	Contamination. Primer dimers.	Use fresh reagents. Redesign assay.
C_t recorded in –RT control.	DNA contamination.	DNase treat samples.
RNA dilution standard curve is not linear.	RT was performed using oligo-dT or random primers.	Use specific priming or dilute cDNA for standard curve.

TABLE 3 | Troubleshooting table (continued).

Problem	Possible reason	Solution
SYBR Green I		
No increase in fluorescence with cycling.	A reagent is missing from the PCR reaction.	Gel analyze PCR product to determine if there was successful amplification and repeat the PCR.
	The $MgCl_2$ concentration is not optimal.	Increase up to 5.0 mM in 0.5 mM increments.
	Hot-start DNA polymerase was not activated.	Ensure that the appropriate initial incubation at 95 °C was performed as part of the cycling parameters.
	Is SYBR Green I concentration too high?	Ensure the correct dilution of SYBR Green I was used.
	Too high a template concentration was used.	Dilute 1 in 10^5 and repeat PCR.
	Insufficient annealing and extension times.	Check the length of the amplicon and increase the extension time if necessary.
	Too few cycles in the PCR reaction.	Increase cycle number; redesign more efficient assay.
	Inappropriate annealing temperature.	Reduce annealing temperature.
Multiple peaks in melt curve.	Abundance of primer-dimer and nonspecific PCR products.	Increase the annealing temperature; lower the Mg^{2+} concentration; always use a hot start polymerase; design more optimal primers.
	Amplification of splice variants/pseudogenes.	Analyze products on gel; sequence different size bands; redesign primers.
Probe		
No increase in fluorescence with cycling.	The probe is not binding to the target efficiently because the annealing temperature is too high.	Verify the calculated T_m, using appropriate software. Note that Primer Express T_ms can be significantly different than T_ms calculated using other software packages.
	The probe is not binding to the target efficiently because the PCR product is too long and is folding in solution.	Design the primers so that the PCR product is no longer than 120.
	The probe is not binding to the target efficiently or being cleaved effectively because the Mg^{2+} concentration is too low.	Perform a Mg^{2+} titration to optimize the concentration.
	The probe has a nonfunctioning fluorophore.	Verify that the fluorophore functions by detecting an increase in fluorescence in the denaturation step of thermal cycling or at high temperatures in a melting curve analysis. If there is no increase in fluorescence, redesign and/or resynthesize probe.
	The reaction is not optimized and no or insufficient product is formed.	Verify formation of enough specific product by gel electrophoresis.

ANTICIPATED RESULTS

Standard curve

Analysis of the data from the standard curve can provide a substantial amount of information about the assay. For this reason, assays should be initially validated on a serial dilution of high quality template even in situations where data collection will not

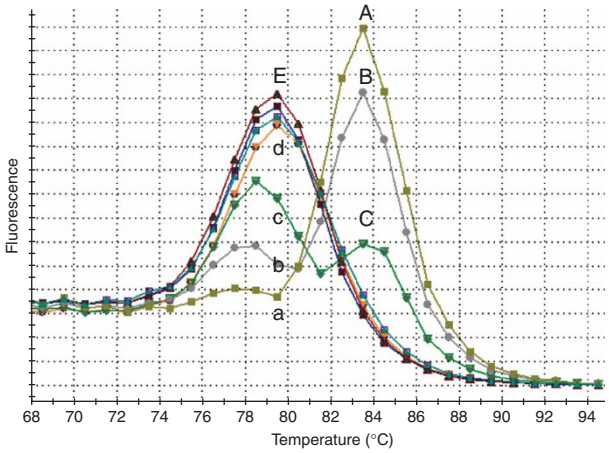

Figure 11 | SYBR Green melt curve demonstrating the appearance of primer dimers in a poorly designed assay. At high RNA concentration (50 ng), a single melt curve appears at around 83 °C (trace A), with no primer dimer (trace a). Two serial 10-fold dilutions result in a reduction in the 83 °C peak (B,C) and the appearance of a second peak at around 79 °C (b,c). Further dilution results in the absence of any target-specific amplification and an increase in the nonspecific peak (d). The position of peaks b–d is identical to the no-template control (NTC) (E).

require reference to a standard curve. The template material used to generate a standard curve should accurately reflect the sample complexity and this means using a total RNA preparation, either from the tissue sample under investigation or from a commercially supplied reference RNA, or spiking a known amount of cDNA, plasmid DNA or oligonucleotides into a tRNA solution. Each concentration should be amplified in triplicate to allow a determination of reproducibility.

The standard curve is constructed from a measure of C_t (y-axis) against log template quantity (x-axis); the C_t of unknown samples can then be compared to this curve to determine the amount of starting template. On the instrument software, use the plate set up facility and define the appropriate wells as "standards" and specify the concentration in those wells. The standard curve is then constructed automatically. It is realistic to expect a linear dynamic range of at least 6 logs with highly reproducible quantification, with >9 logs feasible (**Fig. 7**). This defines the working dynamic range for the assay. One measure of assay efficiency is made by comparison of the relative C_t values for subsequent dilutions of sample. The efficiency of the reaction can be calculated by the equation: $E = 10^{(-1/\text{slope})} - 1$. The efficiency of the PCR should as close to 100% as possible, corresponding to a doubling of the target amplicon at each cycle. Using this measure an assay of 100% efficiency will result in a standard curve with a gradient of –3.323 (also see http://www.gene-quantification.de/efficiency.html). An optimized assay will result in a standard curve with a slope between –3.2 and –3.5. Reproducibility of the replicate reactions also reflects assay stability, with R^2 values of 0.98 or above being indicative of a stable and reliable assay. The intercept on the C_t axis indicates the C_t at which a single unit of template concentration would be detected and is therefore an indication of the sensitivity of the assay (**Fig. 7**).

Figure 12 illustrates typical results obtained for amplicons detected using SYBR Green I (a) and TaqMan (b) chemistries. **Figure 12b** illustrates typical results for amplicons detected using TaqMan chemistry.

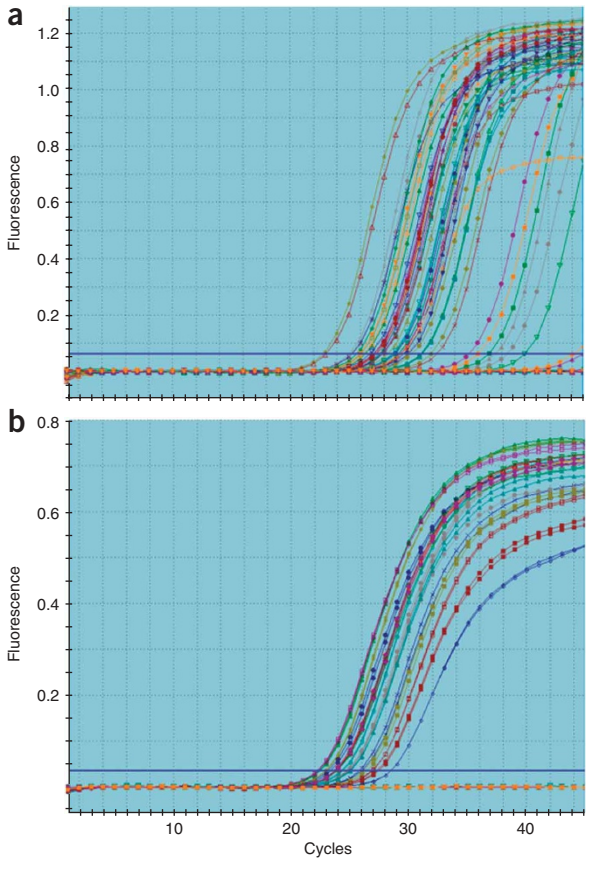

Figure 12 | Comparison of SYBR Green I and TaqMan amplification plots. (**a**) Typical amplification plot obtained using a SYBR Green I assay. Note that the slopes of all amplification plots are identical, indicating that the amplification efficiencies of every sample are the same. Also note the high ΔR_n value typical of SYBR assays. One of the plateaus shows a significantly reduced ΔR_n value, but this clearly does not interfere with the ability to quantitate accurately from that sample. Also note that the NTCs (the two right-most amplification curves) are showing positive C_ts of 44.2 and 45, respectively. This phenomenon is fairly common with SYBR Green assays and is due to the formation of primer dimers in the absence of template. In this example it would be acceptable to increase the threshold line (blue), which would result in negative NTCs[4]. However, we would suggest that the presence of the primer dimers is reported, together with the ΔC_t between the highest C_t recorded by an unknown and the NTC. (**b**) Typical amplification plot obtained using a TaqMan assay. The replicates show a good standard of pipetting and all of the amplification plots have the same slopes, indicating identical amplification efficiencies. The ΔR_n values are significantly lower than those recorded for SYBR Green, and are probe-dependent: the better the quenching, the higher the ΔR_n. Note that in this example the NTCs do not give rise to an amplification curve crossing the threshold, hence are absent, since the probe will not detect primer dimers. If NTCs are positive in a probe-based assay, this is usually due to contamination and it is best to repeat the assay, unless the ΔC_t between the least abundant unknown and the NTC is >5.

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