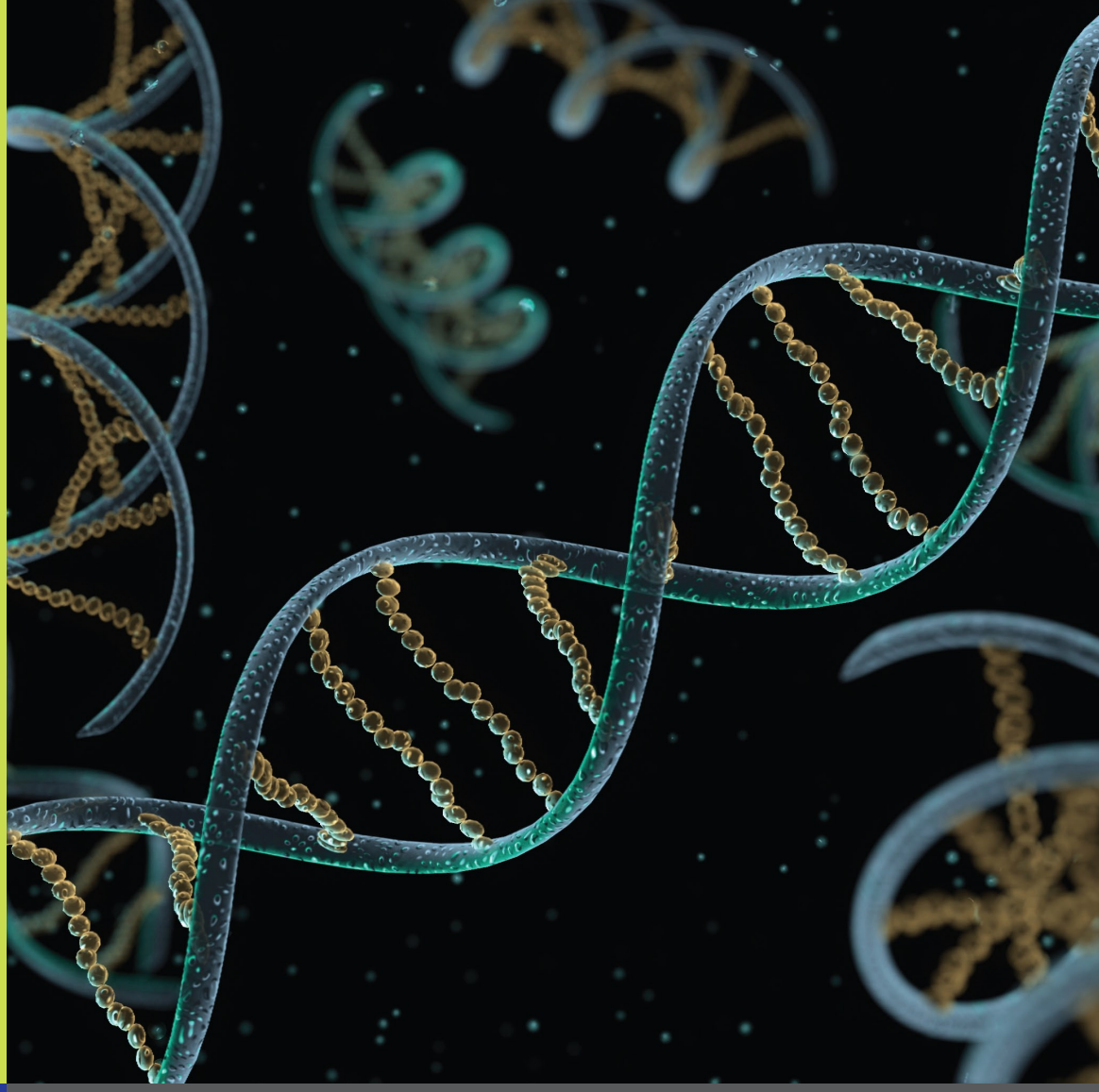

The Future of qPCR Webinar Q&A

Best Practices, Standardization and the MIQE Guidelines

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The tremendous interest and viewership of the webinar "The Future of PCR: Best Practices, Standardization and the MIQE Guidelines" resulted in many questions that were asked by the viewers. These ranged from very specific experiment-level questions, to those that were about the broader qPCR topic. These questions were reduced to the most frequently asked with the broadest applicability and were categorized into several topic areas, as detailed below.

Responses compiled and edited by the panelists: Dr. Stephen Bustin, Dr. Gregory L. Shipley and Manju Sethi

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Webinar hosted by ***Science/AAAS*** and sponsored by Thermo Scientific NanoDrop and Solaris Products
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The material included for each topic is not intended to be a complete discussion of the subject, rather to address and provide guidance to specific questions.

Contamination
- What contaminants are typically associated with qPCR experiments?
- How can they be detected?
- How can contaminants be eliminated?

C_q
- What is an acceptable or reliable C_q?

Data analysis
- What are some common data-analysis software options?
- Are there any databases for real-time data (like GEO ARRAY Express for microarray)?
- What is the recommended procedure if the SD is too high to get a P value?

Efficiency
- What was the significance of the 93% efficiency, referenced in the talk?
- What does it mean if the efficiency is over 100%?

LOD
- How is LOD calculated?
- How can one assess if variation is significant or not?

MIQE Guidelines
- Where can the MIQE Guidelines be found?
- What do the guidelines include?
- To what are they applicable?
- What should be included when publishing?
- How does one use the guidelines?

Normalization Genes
- How does one select reference genes for complex samples, e.g., soil?
- What if the expression levels of normalization genes are variable?
- How many reference genes are recommended?
- Should reference genes be included on every plate?

Primers/Probes/Controls
- What are the main considerations for primer design?
- Where can one find the best primer sequences being used?
- Can a primer/dimer be ignored?
- How should primers be stored?
- What is the advantage of a quencher?
- What is preferred — binding to a "-" or "+" strand?
- What is preferred — sitting on forward or reverse primer?
- What procedure is recommended when there is non-specific binding in the control?

qPCR Applications
- Can qPCR be used for food product testing?
- Can qPCR be used for plant materials?
- Can qPCR be used for clinical microbiology?

Quantification and Purity
- What does one quantify with a NanoDrop?
- What is the importance of the 260/230 ratio and what are the criteria?
- Will Phenol / TE affect an experiment?
- How can one improve RNA quality?

Standard Curves and Delta Delta C_q
- What is best to use — plasmid DNA or Oligo ssDNA for standard curves?
- Should the standard curve be included in the published data?
- Is there a suggested number of points?
- Should a standard curve be run on every plate?
- When should one use Delta Delta C_q?

SYBR Green®/TaqMan®
- Can both SYBR Green and TaqMan be used in one tube?
- Is an NTC control needed for each sample?
- Are there any problems with multiplexing, if amplicons have different melting points?
- What is the importance of the length of amplified fragments?
- Can results from different instruments be combined/averaged?

Contaminants

Contaminants in a sample being prepared for qPCR could be residual genomic DNA, contaminants not removed by the extraction procedure, or chemical contaminants introduced by the extraction procedure itself.

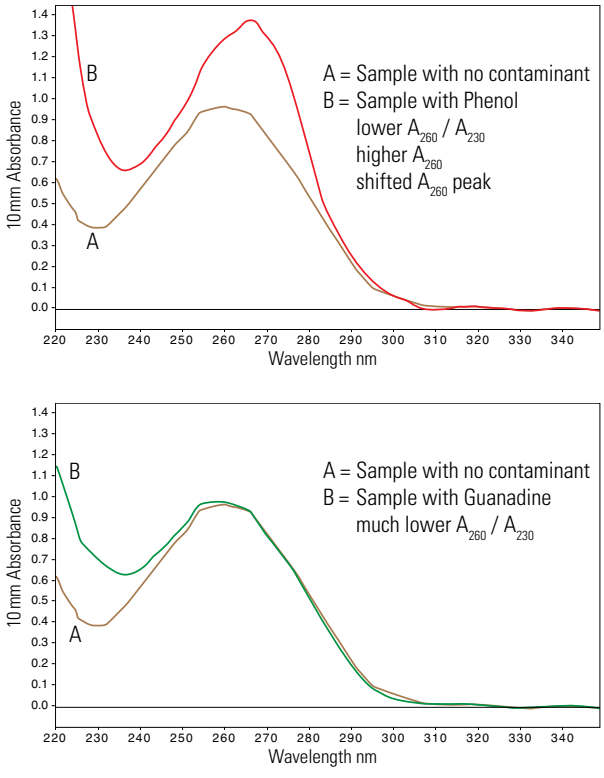
(1) Genomic DNA contamination
Some studies have shown that presence of genomic DNA can shift C_q values significantly. This is particularly so for genes with low expression levels.

There are two options to address this problem:

(a) Removal of the DNA contamination from RNA preparations using commercially available kits that contain RNase-free DNase.

(b) When possible use primers spanning an exon-exon junction. The result, genomic DNA, even when present, is not amplified.

(2) As a result of extraction procedures (conventional extractions or kit-based), there are a number of chemical contaminants that may be present in the sample. Examples of these are Phenol, Guanidine (from extraction), or TE, EDTA (from buffer). The effect of these contaminants on downstream applications may vary. It is always good practice to remove contaminants, so that you are working with a completely known sample for processing through the downstream applications. This practice minimizes parameters that may introduce variability in the final data.



Effect of common contaminants (Phenol and Guanadine) on DNA absorbance spectra.

To remove Phenol residue from the sample, the recommendation is to increase the wash steps. For residues left by kits, the kit manufacturer often supplies separate cleaning agents that will address the removal of these contaminants.

The presence or absence of many common chemical impurities such as those listed here can be verified by the A_{260}/ A_{230} ratio and spectral data from the Thermo Scientific NanoDrop 2000 series spectrophotometer. (See the section on Quantification and Purity Assessment).

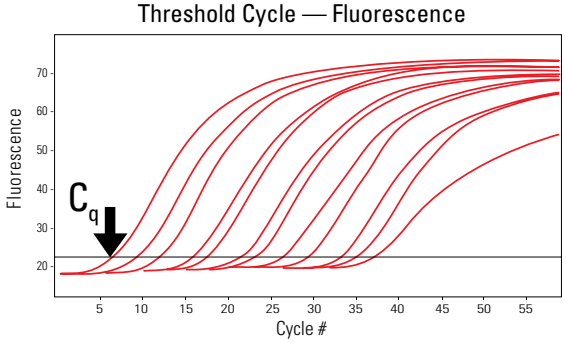
C_q

Those new to qPCR may ask what is considered an "acceptable" or "reliable" C_q. There is no universal answer to this question, if the technique is performed properly.

All qPCR experiments start with the assay. The assay must be validated before unknown samples are run. During this validation both the slope and Limit of Detection (LOD) are determined. The LOD typically is assumed to be the highest C_q value observed for a truly positive sample, as verified by melt-curve analysis or electrophoresis.

The Limit of Detection is used to determine what the acceptable C_q values for that assay are. It may be different for every assay.

There are actually two terms to be considered for qPCR, the LOD or limit of detection and LOQ or limit of quantification (Linnet, *et al.* 2004, Clinical Chemistry **50**:732-740). There are times when one can detect a good signal with an assay at low template concentrations, but that signal will not be within the linear range of the assay. Thus, the LOD will have a higher C_q value than the LOQ. It is the LOQ that must be used as the lower limit for that assay. This can be determined by making a 7-$\log_{10}$ dilution curve of PCR product. First, make a 1/10,000 dilution of a 40 cycle reaction. Use a carrier such as nuclease-free tRNA at 30 – 100 ng/µl, as even dsDNA will stick to plastic at low concentrations. The highest dilution of template that is still linear is the LOQ. One can detect a good signal at lower template concentrations. However, if the signal does not fit on the linear regression line, that data point is no longer useful for data quantification. The result will be a higher C_q for the LOD than for the LOQ. Use the C_q value of the LOQ for a lower limit for that assay.



Threshold cycle (C_q) is the point where the instrument first detects fluorescence above background noise (arrow).

Data Analysis

Data analysis refers to the evaluation of the data with respect to quality and reliability, to ensure that the results can be duplicated and published with confidence. To accomplish this, there are several commercially available software packages. Since there is evidence that qPCR data-analysis methods differ substantially in their performance[1], the selection of the appropriate data collection and processing strategy is important. It should be based on the individual experimental objectives and parameters.

Below is a partial list of common data analysis software programs:

- qBaseplus
- GenEx
- REST-2009
- DataAssist

There are also some integrated software LIMS solutions focused on the life science lab (e.g., Genologics) that help to manage data. A database specifically for real-time qPCR data is qPCR-DAMS: **http://www.gene-quantification.de/jin-bioinf-qpcr-dams-2006.pdf** At this time, we do not have sufficient information to assess its value or quality.

Efficiency

Individual samples generate different and individual fluorescence histories in kinetic RT-PCR. The shapes of amplification curves differ in the steepness of any fluorescence increase and in the absolute fluorescence levels at plateau, depending on background fluorescence levels. The PCR efficiency has a major impact on the fluorescence history and the accuracy of the calculated expression result. It is critically influenced by PCR reaction components. ***Efficiency evaluation is an essential marker in gene quantification procedure***. Constant amplification efficiency in all compared samples is one important criterion for reliable comparison between samples. This becomes critically important when analyzing the relationship between an unknown sequence, versus a standard sequence, which is performed in all relative quantification models.

In experimental designs employing standardization with housekeeping genes, the demand for invariable amplification efficiency between target and standard is often ignored, despite the fact that corrections have been suggested. A correction for efficiency, as performed in efficiency-corrected mathematical models, is strongly recommended. It results in a more reliable estimation of the 'real expression ratio,' compared to no efficiency correction. Small efficiency differences between target and reference genes generate a false expression ratio, resulting in the researcher over- or under-estimating the actual initial mRNA amount. The software listed above can correct for efficiency differences between different assays for analysis.

The assessment of the exact amplification efficiencies of target and reference genes must be carried out before any calculation of the normalized gene expression is executed.[2]

Efficiency is calculated as follows:

$E = 10[-1/\text{slope}] - 1$

For those using the Delta Delta C_q (dd C_q) method, when two assays have differing slopes, the C_q values for each result in different numbers of template for each assay. Thus, when you compare dd C_q from one assay to another, the values will not be the same as if you had used a standard curve to determine a copy number for each assay. This is problematic when the assay(s) used for data normalization do not have the same PCR efficiencies as the experimental assays. When you use the standard curve method, the curve is generated by the same assay as the unknowns. Thus, the copy numbers resulting from the curve take the slope into account. This method has greater accuracy than using mass, which does not take into account the length of the PCR amplicon. Therefore, the copy numbers from assays with different PCR efficiencies are directly comparable, because log-transformed copy numbers account for the different slopes.

Using a synthetic standard (DNA oligo) has its advantages because it is relatively easy to have made and mimics the cDNA exactly. One could also use an in vitro transcribed RNA as template. However, quantification of these RNA standards requires more than just making a A_{260} measurement as not all of the product will be usable in the qPCR assay. A linearized plasmid construct or PCR product may also be used if it is ensured that the dsDNA vs. ssDNA issue is taken into account for quantification.

However, standard curves are not perfect. They will vary slightly from run to run due to pipetting differences in making the dilutions and in adding the standards to the reactions. A compromise would be to use a single standard concentration with a known copy number on each plate and use this C_q value for data analysis.

Limit of Detection (LOD)

LOD, actually the LOQ (see C_q discussion) is determined by taking multiple 10-fold dilutions of PCR product and determining when the assay stops being linear. That is, the lowest point will fall off the line by not working at all. Or, it will not be efficient enough to be co-linear with the lower dilutions. A dilution that gives a signal but is no longer co-linear with the other dilutions is the LOD. However, it's preferable to use the LOQ as the C_q value cut-off for the assay. It is suggested that a carrier is used to make the dilutions, such as nuclease-free tRNA (yeast or *E. coli*). This will keep the lowest dilutions from sticking to the plastics. Assay quality is measured by PCR efficiency, and it is a user decision on how low this can be. If the LOQ of the assay is sufficient for the samples, the PCR efficiency can be poor, (less than 93% or a slope of –3.5). Poor PCR efficiencies tend to give higher LOQ values. Although the assay works well enough for this sample set, it may not work well for the subsequent set. The primers are the key to a good assay. Changing a primer up or down in the template sequence by just one base may make a significant difference in the PCR efficiency.

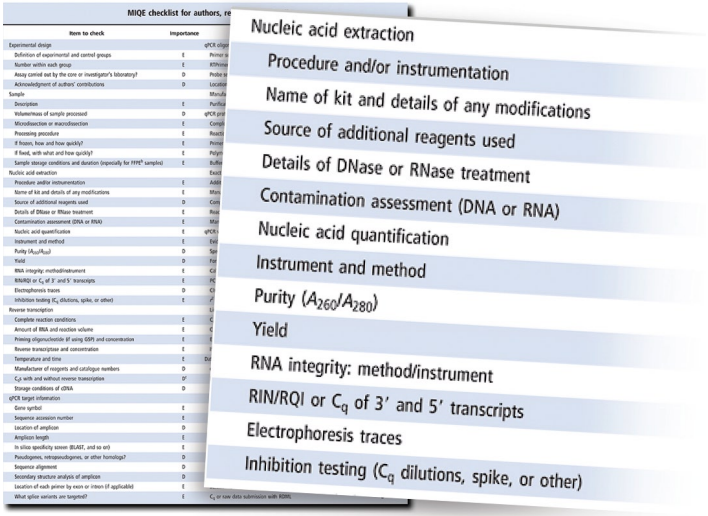
For example, at the Quantitative Genomics Core Lab at the University of Texas Science Health, Houston, the general rule for design of qPCR assays is greater than 93% PCR efficiency and linear down to 10 copies of template. To achieve this goal, 4 primers are ordered for each assay, 2 forward and 2 reverse, as primers are very inexpensive. They are tried in all 4 combinations, to find the best pair. Software is good but not perfect. Determining what is 'best' is difficult, as there is no basis for comparison.

MIQE Guidelines

The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments is published in the *Journal of Clinical Chemistry* 611-622 (2009).

It provides guidance for a standardized way to conduct and report experimental details of a qPCR experiment to "help ensure the integrity of the scientific literature, promote consistency between laboratories, and increase experimental transparency."

Importantly, it includes a practical checklist to accompany the initial submission of a paper to the publisher.



MIQE Guidelines checklist

Irrespective of whether the intended purpose of the qPCR experiment is publishing, the MIQE Guidelines provide a disciplined approach to conducting qPCR. It provides added confidence in the resulting data, and the ability to reproduce the results reliably.

While there are no strict rules for what to include in the publication, the following provides some suggestions in addition to those included in the MIQE Guidelines checklist:

1 – Species

2 – Accession number

3 – Synonyms (old names for the same transcript)

4 – Primer/probe sequences with strand designations (strand to which the primer binds, + or -), reporter (e.g., FAM) and quencher (e.g., BHQ1) IDs

5 – PCR amplicon length

6 – PCR efficiency (in %)

7 – Exon boundary information (e.g., which exon(s) are detected, where the assay lies or if the transcript isn't spliced)

8 – Splice variants (e.g., if they exist, does the assay see them all, only 2 out of 3, only 1, etc.)

9 – Folding issues via M-fold (e.g., are stems an issue where the primers/probe bind in the PCR amplicon)

Normalization Genes/Controls

Data normalization in real-time RT-PCR is a further major step in gene quantification analysis (Bustin 2002, Pfaffl 2001). The reliability of any relative RT-PCR experiment can be improved by including an invariant endogenous control (reference gene) in the assay. This will correct for sample-to-sample variations in RT-PCR efficiency and errors in sample quantification. A biologically meaningful reporting of target mRNA copy numbers requires accurate and relevant normalization to some standard and is strongly recommended in kinetic RT-PCR. **The quality of normalized quantitative expression data cannot be better than the quality of the normalizer.** Any variation in the normalizer will obscure real changes and produce artifactual changes (Bustin 2000). Real-time RT-PCR-specific errors in the quantification of mRNA transcripts are easily compounded with any variation in the amount of starting material between the samples. For example, it can be caused by sample-to-sample variation, variation in RNA integrity, RT efficiency differences and cDNA sample loading variation (Stahlberg 2003 2004a 2004b). This is especially relevant when the samples have been obtained from different individuals, different tissues and different time courses. It will result in the misinterpretation of the derived expression profile of the target genes. Therefore, normalization of target gene expression levels must be performed to compensate the intra- and inter-kinetic RT-PCR variations, such as sample-to-sample and run-to-run variations (Pfaffl & Hageleit 2001).

Data normalization can be carried out against an endogenous, unregulated reference gene transcript or against total cellular DNA or RNA content (molecules/g total DNA/RNA and concentrations/g total DNA/RNA). Normalization according to the total cellular RNA content is increasingly used, but little is known about the total RNA content of cells or about the mRNA concentrations. The content per-cell or per-gram tissue may vary in different tissues in vivo, in cell culture (in vitro), between individuals and under different experimental conditions. Nevertheless, it has been shown that normalization to total cellular RNA is the most reliable method (Bustin 2000 Bustin 2002). It requires an accurate quantification of the isolated total RNA or mRNA fraction by optical density at 260 nm, Agilent Bioanalyzer 2100, or Ribogreen RNA Quantification Kit. Alternatively, the rRNA content has been proposed as an optimal and stable basis for normalization, despite reservations concerning its expression levels, transcription by a different RNA polymerase and possible imbalances in rRNA and mRNA fractions between different samples (RNA and RT page).

To normalize the absolute quantification according to a single reference gene, a second set of kinetic PCR reactions has to be performed for the invariant endogenous control on all experimental samples. The relative abundance values are calculated for internal control as well as for the target gene. For each target gene sample, the relative abundance value obtained is divided by the value derived from the control sequence in the corresponding target gene. Then, the normalized values for different samples can be compared directly (Pfaffl 2001, relative expression page).[3]

In many instances, transcripts that are found in multiple cell types are selected as the reference genes, since they can be applied to any qPCR experiment. In practice however, any transcript can be used for data normalization. Finding which one to use for a particular assay is an

Normalization Genes/Controls *continued*

empirical process that cannot be avoided. There are commercial arrays available for finding suitable transcripts for data normalization. One can also try a cadre of accessible transcripts.

The number of reference genes to use depends on the variability seen with just one, then two, then three and so on. Programs such as GeNorm, qBasePlus or GenEx have functions that allow one to make this assessment. Furthermore, it has been shown that using multiple transcripts for data normalization can result in a better analysis (Vandesompele, *et al*. 2002, Genome Biol **3**:RESEARCH0034).

In the case of complex matrices (e.g., soil, blood), such as a loss of cellular context, or when there is a rapid change in normal transcript levels (e.g., apoptosis or ActD induced transcript half life measures), it is best to use total nucleic acid measures for normalization. In principle, the cutoff is determined by the LOQ of each individual assay. The C_q values that determine the LOQ can be different for every assay. Therefore, it is not a safe assumption to say that the same cutoffs may be used for target and normalization genes.

Primers/Probes/Controls

PRIMER

Optimal primer length is typically 15 – 25 bases, and the GC content can vary between 40 – 70%. It is recommended that the two primers should have closely matched melting temperatures, and the difference should be ≤ 5˚C. Generally, primers with melting temperatures in the range of 55 – 65˚C will produce the best results. To minimize mis-priming, primers should be designed with no more than 2 G/C bases in the last five bases, at the 3' end of the primer. Formation of primer-dimers can be limited by avoiding primers with secondary structures or complementary sequences between the 3' ends of forward and reverse primers and for RT-qPCR. Ideally, primers should be designed to span the exon-exon boundary.

Degenerate primers can be used. However, extra care must be taken to check the results, as it is difficult to predict the performance of degenerate primers solely based on the sequence.

The formation of primer-dimers can reduce the amplification efficiency of the specific target. In this case, it may be best to design more suitable primers. Use of TE buffer (10 mM Tris•Cl, 1 mM EDTA, pH 8.0) is recommended. For standard primers, making a concentrated stock solution (e.g., 100 µM) and storing in small aliquots at -20˚C is recommended. Standard primers are stable under these conditions for at least one year. Freeze-thaw cycles should be avoided since they may lead to degradation. Primers should not be dissolved in water, since they are less stable in water than in TE. Additionally, some primers may not dissolve well in water.

Validated PCR primer sequences may be submitted to the following database: The link is: **http://medgen.ugent.be/rtprimerdb/**

PROBE

Quenchers are useful in multiplexing assays. There is usually a higher signal-to-noise ratio with non-fluorescent quenchers (like BHQ), compared to reporters used as quenchers (e.g., TAMRA). Probes should be designed to match to the fluorophore. If you use fluorophores as quenchers you also "lose" one detection channel for it.

There is little difference between binding to the "-" or "+" strand, if the primer is designed properly.

Similarly, it does not matter significantly if the probe sits on the forward or reverse primer, so long as one finds an optimal place.

CONTROL

Non-specific binding of controls is often related to unspecific primers, too low of an annealing temperature or contamination. If one uses the universal 60˚ – 95˚C cycling protocol, it may be best to redesign the primers or probe, or to optimize the annealing temperature to alleviate this problem.

The issue of a signal from no template controls (NTC) could be from primer-dimers or from contamination with SYBR Green chemistry. If the control is a positive control (known amount of target DNA), and there is unspecific binding, it may be attributable to primers that are non-specific and allow annealing to other targets.

At the Quantitative Genomics Core Lab at the University of Texas Science Health, Houston, the following practices are followed. Primers/probes are dissolved in nuclease-free H_2O with no problems. 100 µM stocks are made as stated above and stored at –80˚C. For working stocks, 20 µM primer/probe dilutions are made and stored at –20˚C. In fact, primers and probes have been seen to be good after 10 years of storage under these conditions. However, as mentioned above, repeated freeze/thaw cycles will have an affect on primer/probe stability.

The assays at this facility are designed for the primers to have an optimal binding Tm of 58˚ – 59˚C and to keep the Tm difference among primers at 2˚C. A 5˚C Tm may be too large of a difference, if the annealing temperature of the reaction may have to be altered for assay optimization. All of the assays at this facility use a 60˚C annealing/extension temperature.

As for primer probe structure, runs of Gs over 3 in a row should be avoided, as this sequence will tend to put a bend in the oligo that can affect hybridization. Select probes based on the annealing strand that will minimize the number of Gs in the probe sequence. It may be beneficial to keep the probe close to the primer that anneals to the same strand as the probe. Using good software to make your assay design is crucial.

qPCR and its Applications

PCR "is one of a handful of techniques (perhaps ***the*** technique) that have truly revolutionized molecular biology. Its specificity, efficiency, and fidelity have turned it into a key technology that has made molecular assays accessible to every research and diagnostic laboratory. It underpins most of the spectacular advances that are now commonplace in every biological discipline, ranging from microbial detection and microbiological quality assurance, through the detection of genetically-manipulated organisms in crops and foods, to molecular and veterinary medicine. Together with its offshoot, the reverse transcriptase (RT) PCR, it is unrivalled as a quantitative assay for the rapid, inexpensive and simple detection of nucleic acids."[2]

While conventional PCR is a qualitative assay (answering yes/no questions), quantitative, real-time PCR (qPCR) is a powerful technique that enables both qualitative as well as quantitative measurements of specific genes in a nucleic acid sample. Since various experimental parameters can have a significant impact on the quality of results (in some cases erroneous but believable), it is particularly important to employ standardized best practices. Those include the use of rigorous controls, validation and non-subjective data interpretation.

A partial list of key applications is shown below:

Molecular Diagnostics

e.g., Quantification of viral loads in clinical samples
Solid tumor diagnostics
Identification of specific genes that dictate therapeutic outcome

Life Science Research (including Plant/Animal)

e.g., Gene Expression
SNP analyses
Detection of genetic alterations
Cloning

Biotechnology

e.g., Quantification/Detection of genetic modification in foods (GMOs)

Food

e.g., Quantification/Detection of bacterial pathogens

Quantification and Purity Assessment of Samples

"The traditional method for measuring DNA concentration is the determination of the absorbance of UV light at 260 nm (A260) in a spectrophotometer …"[1]

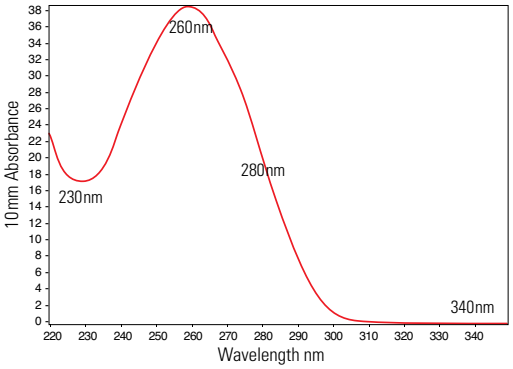
Typically quantification is done after DNA/RNA extraction. If reverse transcription is performed using the same RT kits and with the same sample type, it is not necessary to quantify the cDNA post RT.

The Thermo Scientific NanoDrop 2000 series is an easy-to-use, microvolume, UV-Vis spectrophotometer that allows samples to be measured quickly (less than 5 seconds) and conveniently, without the need for cuvettes. It utilizes a patented sample-retention technology to measure samples as small as 0.5 µl – 1.0 µl, without the need for dilutions. An IQ/OQ kit is also available, which includes procedures and forms useful for the validation of the instrument for labs that require such practices.

The NanoDrop™ 2000/2000c provides the following information for a sample:

- DNA/RNA concentration (quantification)
- Purity assessment using A_{260}/ A_{280} and A_{260}/ A_{230} ratios
- Full (absorbance) spectral data

Historically, A_{260} /A_{280} has been the primary measure of purity. Users are now gathering more information by examining the entire absorbance spectra, including A_{260} /A_{230} .



Absorbance spectrum for DNA/RNA

UV-Vis absorbance measurements do not distinguish between RNA and DNA (both absorb at 260 nm). It assumes that the sample being measured is pure. (See section on Contaminants for more information on removing genomic DNA from RNA samples).

Quantification

The dynamic range of the NanoDrop 2000 series is 2 ng/µl – 15,000 ng/µl (dsDNA) or 2 ng/µl – 12,000 ng/µl (RNA) — without the need for dilutions. In addition to the microvolume sample retention system, the NanoDrop 2000c model also provides a built-in cuvette capability, which extends the detection limit down to 0.4 ng/µl (dsDNA).

Purity

Recommended A_{260}/ A_{280} ratios are:
For pure DNA: ~ 1.8
For pure RNA: ~ 2.0

Recommended A_{260}/ A_{230} ratios are:
For pure DNA: ~ 1.8 – 2.2
For pure RNA: ~ 1.8 – 2.2

In some cases, the above ratios for an *impure* sample may be in the acceptable range, so it is important to examine spectral data in conjunction with these ratios. The sample spectrum can provide additional information regarding potential chemical contaminants introduced by extraction procedures or kits. (See the section on Contamination for more information on removing chemical contaminants).

Standard Curves/Delta Delta C_q

The two most commonly used methods to analyze data from real-time, quantitative PCR experiments are absolute quantification and relative quantification.

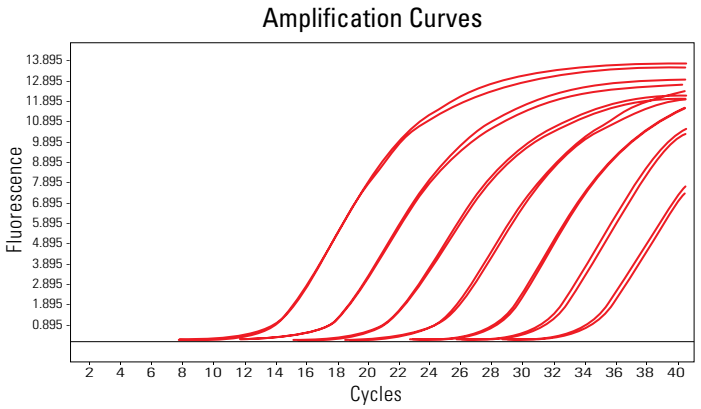
Standard Curves

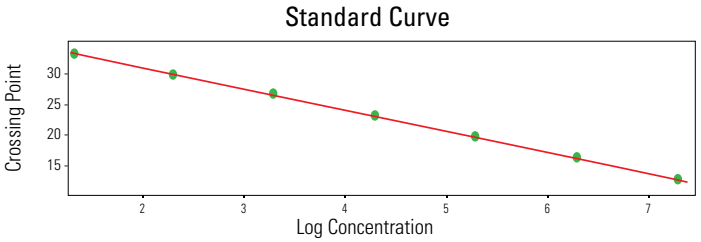
Absolute quantification determines the input copy number and is usually accomplished by comparing the sample PCR signal to a standard curve.

To construct the standard curve, any template that can be specifically amplified by the qPCR assay components may be used, e.g., ssDNA oligo standards that are the same length as the PCR amplicon. If dsDNA standard is used, there is a two-fold reduction in copy number that will result from the comparison. This is because cDNA is ssDNA, and the first cycle for ssDNA is making the dsDNA with no amplification. Whereas with dsDNA, the amplification starts at cycle 1.

In order to mimic total RNA in the dilutions of the standards, one can use molecular-biology grade tRNA from yeast or E.coli at 30-100 ng/µl. A carrier is used to dilute the standard material, which prevents low copy number molecules from sticking to the plastics.

There is no mandated number of dilution points required for a standard curve. Ideally, one would need to dilute down to 1 copy to define the LOQ. Since this is not practical, typically, 5 -10 copies will allow for determining a realistic LOQ. It is critical not to use a C_q value higher than the LOQ for an experiment. Extrapolation is not recommended under any circumstances. Typically, 7 logs of dilution are required to define the LOQ. If a standard curve is run with the unknown samples, a 5-log standard curve in duplicate is sufficient. With PCR product, this cannot be determined easily without dilution. The recommendation would be to make a 1/10,000 dilution and to start from there with 7 logs, each in duplicate. This only has to be done once for each assay. However, in order to have maximum confidence regarding PCR reagents and cycling conditions, one may elect to have the standard curve run on every plate (with samples for that assay).





Delta Delta C_q

Relative quantification relates the PCR signal of the target transcript in a treatment group to that of another sample, such as an untreated control. The comparative C_q method (also known the Delta Delta C_q method, the $2^{-[delta][delta]}C_q$ method or dd C_q) is commonly used to compare the C_q values of the samples of interest with that of the untreated control. All (sample and control) C_q values are normalized to an appropriate endogenous housekeeping gene. For the [delta][delta]C_q calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. This can be established by looking at how [delta]C_q varies with template dilution. If the plot of cDNA dilution versus delta C_q is close to zero, it implies that the efficiencies of the target and housekeeping genes are very similar. Technically, the slopes have to be nearly identical for a dd C_q analysis. (Refer to the section on Efficiency).

If a housekeeping gene cannot be found whose amplification efficiency is similar to the target, then the standard curve method may be preferred.

The MIQE Guidelines do not recommend any method. MIQE Guidelines provide guidance in best performance practices for all aspects of a transcript or gene quantification experiment. The quantification approach selected should be based on the requirements of the experiment.

SYBR Green/TaqMan

Overview of TaqMan®- and SYBR®-Green Based Detection[4]

	TaqMan-Based Detection	SYBR-Green Based Detection
Chemistry Overview	Uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles.	Uses SYBR Green I dye, a highly specific, double-stranded DNA binding dye, to detect PCR product as it accumulates during PCR cycles.
Specificity	Detects specific amplification products only.	Detects all amplified, double-stranded DNA, including non-specific reaction products.
Applications	One-step RT-PCR for RNA quantitation Two-step RT-PCR for RNA quantitation DNA/cDNA quantitation - Allelic Discrimination - Plus/Minus assays using an internal positive control (IPC)	One-step RT-PCR for RNA quantitation Two-step RT-PCR for RNA quantitation DNA/cDNA quantitation
Advantages	Specific hybridization between probe and target is required to generate fluorescent signal, significantly reducing background and false positives. You can label probes with different, distinguishable reporter dyes, which allows you to amplify two distinct sequences in one reaction tube. Post-PCR processing is eliminated, which reduces assay labor and material costs.	Enables monitoring of the amplification of any double-stranded DNA sequence. No probes are required, which reduces assay setup and running costs. Multiple dyes can bind to a single amplified molecule, increasing sensitivity for detecting amplification products.
Disadvantages	A different probe has to be synthesized for each unique target sequence.	Because SYBR Green I dye binds to any double-stranded DNA — including nonspecific double-stranded DNA sequences — it may generate false positive signals.

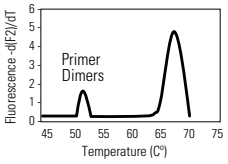
SYBR vs. Probe qPCR Detection

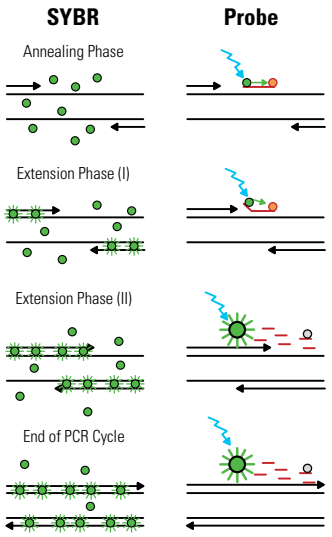
SYBR-based detection

- High-throughput screening approach
- Cannot multiplex
- Binds to any dsDNA formed
- Melt-curve analysis
- Validation of primers

Probe-based detection

- More focused approach
- Binds selectively to target
- Can perform multiplexing
- Few probes allow melt-curve analysis





SYBR Green/TaqMan *continued*

Both TaqMan and SYBR Green may be used in one tube. Naturally, this needs some optimization for primer, probe and dye concentrations. Also, the dyes on the probe cannot use the same channel as the SYBR. Therefore, the instrument used must be calibrated, to ensure proper color separation.

A "No Template Control" (NTC) should be run for each assay to rule out cross contamination of reagents and surfaces. Because PCR is so sensitive, the proper primer pair can result in amplification from even very small amounts of contaminant. Potential contamination includes that which may be inside the pipette or contained in any stock supplies of water, primers, etc. The NTC includes all of the RT-PCR reagents except the cDNA template. Typically, the cDNA is simply substituted with nuclease-free water. No product should be synthesized in the NTC. If a product is amplified, it indicates that one or more of the RT-PCR reagents is contaminated with the target sequence.

When multiplexing using SYBR chemistry (or other dsDNA binding dye), one cannot distinguish the fluorescence generated by the reaction from different amplicons. The only differentiator would be the melting curve, and that does not provide quantitative results. This is because one can only see the "end-point" amount of PCR product. In addition, if the amplicon lengths vary considerably the melting temperatures will vary as well. Typically, shorter amplicons are amplified more efficiently than longer ones. Additionally, if the primer pairs have very different Tm values, annealing temperatures may not be optimal for all of the primers. This may lead to problems. For qPCR, the optimal length of the amplicon is between 50 – 150 bases. Amplification of products over 300 bases is not recommended. Designing primers that generate a very long amplicon may lead to poor amplification efficiency. In cases in which longer amplicons are necessary, optimization of the thermal-cycling protocol and reaction components may be necessary.

It is possible to "compare" results from different qPCR instruments, depending on what results one wants to compare. For example, in absolute quantification, one should not just compare the C_q values of samples given by different instruments. Standard curves need to be run on each instrument and will assess if the C_q values differ. This data then can be used to normalize for the difference. Essentially, all runs need some type of control that is reproducible and can be used to compare different values.

[1] **Statistical significance of quantitative PCR. BMC Bioinformatics 2007; 8:31** Karlen Y. *et al*.

[2] **A-Z of Quantitative PCR** edited by Stephen A. Bustin

[3] **normalisation.gene-quantification.info**

[4] **http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/real-time-pcr/taqman-and-sybr-green-chemistries.html**

http://www.gene-quantification.info/

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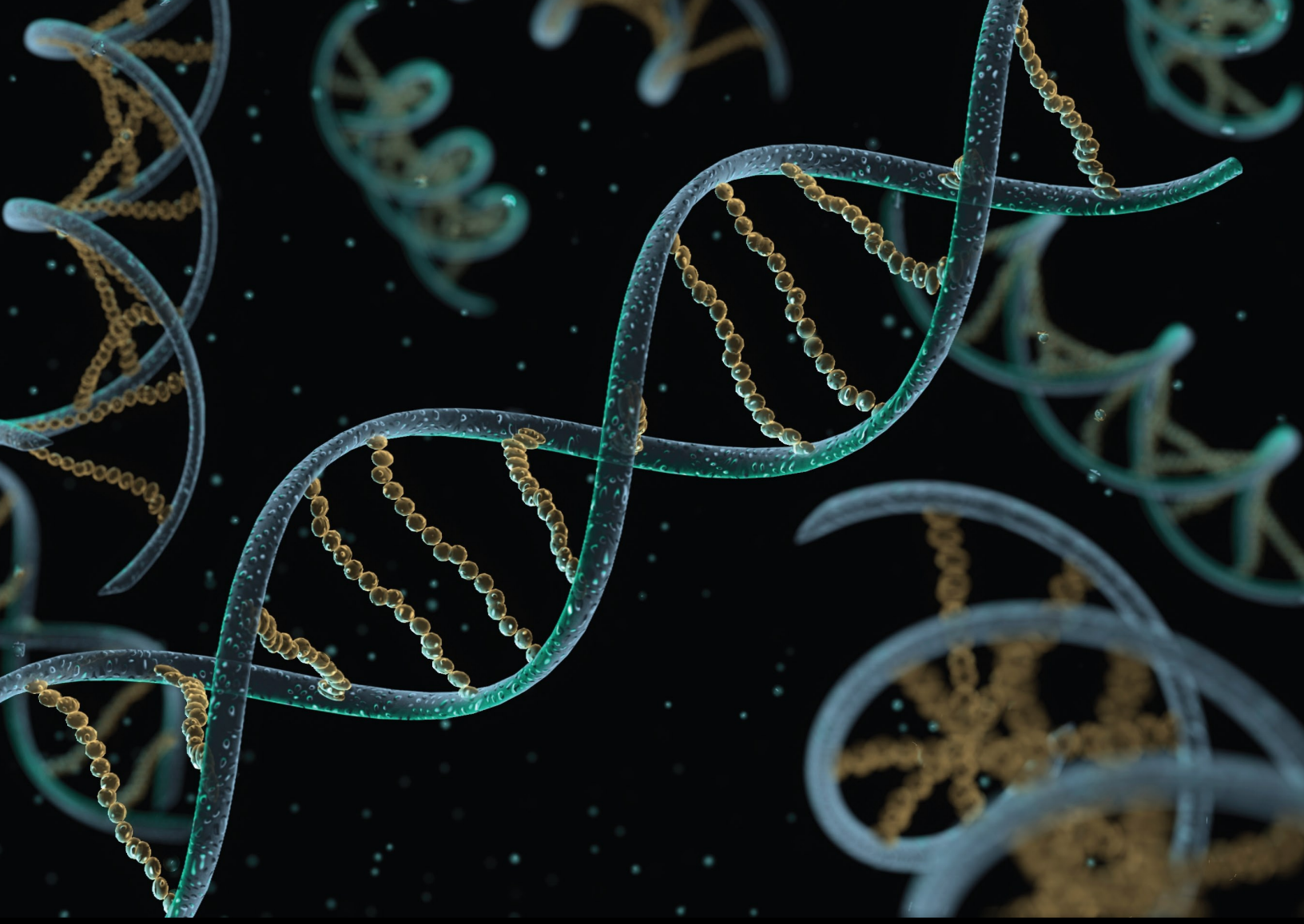

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