National
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System

Good practice guide for the application of quantitative PCR (qPCR)

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Good practice guide for the application of quantitative PCR (qPCR) First Edition 2013

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Foreword by Michael W Pfaffl

The polymerase chain reaction (PCR) is a rapid, sensitive, and rather simple technique to amplify DNA, using oligonucleotide primers, dNTPs and a heat stable *Taq* polymerase. It was invented in 1983 by Kary B. Mullis and co-workers, who, ten years later, were awarded the 'Nobel Prize for Chemistry'. With the introduction of real-time PCR in the late nineties, the PCR method overcame an important hurdle towards becoming 'fully quantitative' (and therefore known as quantitative PCR, or qPCR). Currently, qPCR is regarded as the 'gold standard' in the quantitative analysis of nucleic acids, be it DNA, RNA or micro-RNA molecules. The main reasons for its success are its high sensitivity, robustness, good reproducibility, broad dynamic quantification range, and very importantly, affordability. The assay and primer design can often be fully automated and handling in the lab is blindingly easy.

Another big draw for the user is that, in most instances, the qPCR experiments produce results, or as we call them, Cq data points. However, the generation of Cq data points is not dependent on good laboratory practice or the precise application of guidelines such as MIQE. In other words, when researchers obtain a Cq data point, they need to prove that that particular amplification result is valid, reliable and meaningful.

And exactly here lies the main challenge of qPCR! This method is 'too easy' to apply and generates results any time. It is up to the researcher to demonstrate that the data obtained are valid and if not, investigate where the error could come from.

Hence, it is essential to have a comprehensive understanding of the underlying basic qPCR principles, sources of error, and general issues inherent to nucleic acid isolation and/or quantification in order to develop assays and workflows which meet high analytical requirements in concordance with the MIQE guidelines. Unfortunately, we are still far from having developed such optimal workflows, with the highest sensitivity or the best RNA integrity metrics, to obtain reproducible and authentic results.

Thus, I can warmly recommend to the research community this **Good Practice Guide for the Application of Quantitative PCR**, with the aim to improve researchers' experimental workflows, from sampling to qPCR data analysis, and eventually take us to valid and confident research results.

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Good Practice Guide for the Application of Quantitative PCR (qPCR)

Contents

Objectives of this guide

There is little doubt that PCR (Polymerase Chain Reaction) has transformed the fields of clinical and biological research, due to its robustness and simplicity. Subsequent developments, such as real-time quantitative PCR (qPCR) and reverse transcription qPCR (RT-qPCR), offer simple methods for analysis of DNA and RNA molecules. However, completing qPCR assays to a high standard of analytical quality can be challenging for a number of reasons, which are discussed in detail in this guide.

qPCR has a large number of applications in a wide range of areas, including healthcare and food safety. It is therefore of paramount importance that the results obtained are reliable in themselves and comparable across different laboratories.

This guide is aimed at individuals who are starting to use qPCR and realise that, while this method is easy to perform in the laboratory, numerous factors must be considered to ensure that the method will be applied correctly.

These additional considerations include – but are not limited to – methods of sampling, sample storage, nucleic acid extraction, and nucleic acid storage, manipulation and preparation. In other words, all the steps prior to undertaking the quantification technique must also be controlled. At the other end of the analytical process, reporting technical results may be highly subjective. Since qPCR is a relative method, requiring the comparison of two or more samples to a standard curve or to each other, standardisation of results is very challenging. The primary qPCR metric, the quantification cycle (C_q)[1], depends on many factors including where a threshold is set, the choice of reporter and day-to-day variation in measurement. In addition, since C_q exists on a logarithmic scale, there are specific statistical challenges that need to be addressed to analyse these data accurately.

All these factors combine to make a technically simple technique, challenging to interpret with absolute confidence. This guide aims to assist those who are, or will be, using qPCR by discussing the issues that need consideration during experimental design. The guide entails “tried and tested” approaches, and troubleshoots common issues.

Terminology

Absolute (or Standard Curve) Quantification

Absolute quantification is used when performing qPCR to describe estimation of target copy numbers by reference to a standard curve of defined, absolute concentration. This guide uses the term standard curve quantification to describe this process.

Allelic Discrimination (AD)

Allelic discrimination assays are designed to define and differentiate genetic variants including single nucleotide polymorphisms (SNPs). They use differentially labelled probes (one specific for the wild type and another targeted for the mutant sequence), or a single probe to detect either product followed by a melt curve analysis to distinguish between the two. An alternative approach is to use DNA binding dyes in combination with melt curve analysis (see High Resolution Melt (HRM) analysis).

Amplicon

The amplicon refers to the specific PCR product resulting from amplification of the primer-targeted region.

Amplification Plot

The amplification plot is a graph presenting the relationship between cycle number (*x*-axis) and fluorescence signal (*y*-axis). This results in a sigmoidal curve. Amplification, represented by the initial log phase, is followed by a linear phase, and finally a plateau.

Calibrator

The calibrator is a reference sample used as the basis for quantification studies.

Comparative Quantification

Comparative quantification is used to measure the relative change in expression levels between samples under different experimental conditions or over a period of time. The concentration of the gene target in each sample is compared to a validated reference gene or multiple reference genes, to normalise for variations in sample loading. Comparative quantification is also known as relative quantification.

C_q (quantification cycle)[1]

A generic term which includes $C_{t,}$ (see below), crossing point (C_p), and all other instrument specific terms refering to the cycle used to quantify the concentration of target in the qPCR assay.

C_t (threshold cycle)

The C_t is defined as the number of cycles required to produce a constant emission of fluorescence. The constant fluorescent emission is recorded relative to a defined threshold setting and the cycle number at which the fluorescence generated crosses the threshold is the reaction C_t. (*N.B. The MIQE guidelines[1] propose that C_t be replaced by C_q.*)

End Point Analysis

An end point analysis is used to measure the amount of amplified product at the end of the PCR. Results are considered to be qualitative and are used to indicate the presence or absence of a specific target sequence in a sample without the determination of target concentration.

Endogenous Control/Reference Gene/Normaliser

An endogenous control is typically a gene target (or several in combination) present in each sample at a constant concentration that is resistant to response fluctuations due to changes in biological conditions. These have, historically been referred to as housekeeping genes. However, when measuring RNA, many targets can be used which cannot be considered as housekeeping genes; hence the term of preference is now **reference gene**.

Exogenous Control

An exogenous (or external) control is a target sequence that is spiked into the sample at various stages throughout the measurement process. Measuring the concentration of this spiked sequence can be used as a serial quality control and is widely used to monitor recovery efficiency at different experimental stages, and to identify false negative results.

Gene of Interest (GOI)

The gene of interest is the gene target under investigation.

High Resolution Melt (HRM)

HRM is an extension of the traditional DNA melting analysis (see Melt Curve) and is used to characterise nucleic acid samples based on their dissociation (melting) behaviour. Samples can be defined according to their sequence, length, G:C content or strand complementarity. Even single base changes such as SNPs can be identified. The technique requires a combination of high-intensity optical detection systems, accurate thermal uniformity, high-speed data capture, extreme thermal resolution, DNA binding dyes, and dedicated software analysis.

Melt Curve

The melt curve is a post-PCR analysis performed to estimate the specificity of amplified products based on their melting characteristics. Reactions performed in the presence of double-stranded DNA binding dyes are incubated through a range of increasing temperatures. When the melting temperature (T_m) of the amplicon is reached, the amplicon dissociates from a double-stranded to a single-stranded state leading to a drop in fluorescence of the double-stranded binding dye. Melt curves are presented as a derivative plot showing the rate of change in fluorescent signal. The *x*-axis represents temperature and the *y*-axis displays the negative derivative (rate of change) of fluorescence (F) with respect to temperature (T), shown as dF/dT. The melting temperature is dependent upon the length of the DNA sequence, G:C content and buffer. The peak of the plot represents the melting temperature (T_m) at which ~50% of the DNA is single-stranded.

Multiplexing

Multiplexing is the process of simultaneous amplification and detection of more than one target in a single reaction tube. These reactions are usually detected using gene specific probes with different fluorescent labels associated with each gene target. HRM can also be used to detect multiple amplification products.

No Template Control (NTC)

No Template Control (NTC) qPCRs include all PCR reagents with the exception of the template. This is a standard negative control used to identify set-up contamination and primer-dimer product amplification.

Normaliser or Reference Gene

See Endogenous Control.

One-step Reverse Transcription (RT)-qPCR

Prior to quantification of a target transcript, it is necessary to convert RNA to cDNA. One approach is to perform both the reverse transcription and PCR amplification steps sequentially, in the same tube. In a one-step RT-qPCR, gene-specific primers are used for both the RT and PCR steps.

Passive Reference Dye

The passive reference dye is added to the reaction mix to generate a signal for correction of differences in optical sensitivity between distinct sample tubes. It also provides confirmation that an equal volume of reaction mix has been added to each PCR sample. After baseline correction, the relative concentration of reference dye to signal intensity is traditionally plotted as dRn. The absolute intensity of the reference signal may influence data analysis since it directly affects the signal to noise ratio.

Quantification Cycle (C_q)[1]

See C_q

Quencher

The quencher molecule is positioned in close proximity to the fluorescent label on a dual labelled probe and absorbs the emission of reporter fluorescence, thus sequestering the signal output.

Reaction Efficiency (E) or Amplification Value (A)

The calculated rate of amplification is reported as a percentage, a fraction of 1 (for E) or fraction of 2 (for A). Efficiency calculations assume amplicon doubling during every cycle (for 100% efficiency). Efficiency (E) can be calculated using a standard curve with gradient m, using the following equation (1)[2]:

$$E = 10^{(-1/m)} - 1 \qquad (1)$$

Reverse Transcription

Reverse transcription (RT) is the process of converting RNA to cDNA, using a reverse transcription enzyme and dNTPs.

Reverse Transcription (RT) minus control (RT(-))

The RT(-) control is used to identify contaminating sequences commonly derived from gDNA within a cDNA sample. The RT(-) control sample comprises all the components of the reverse transcription reaction, including target RNA, but with the omission of the reverse transcriptase enzyme. This is another type of negative control (see NTC).

Single Nucleotide Polymorphism – SNP

Single nucleotide polymorphisms (SNPs) are DNA sequence variants, or mutations, at a single base locus.

Standard Curve

A sample (also known as a calibrator) of known concentration units (e.g. pg/μL, copies/reactions, dilution factor, number of cells, or a relative dilution factor) is serially diluted through a controlled series and used to construct a standard curve. The observations or measurements, in this case C_q values of these standards, are plotted against the logarithm of their concentration. The standard curve is used to predict analyte concentration of the unknown test samples from the observed C_q.(see Absolute Quantification).

Threshold Cycle (C_t) Value

See C_t and C_q.

Two-step RT-qPCR

A two-step RT-qPCR is performed as two independent reactions: a reverse transcription reaction followed by qPCR. The reverse transcription step, using a blend of oligo-dT primers and random oligonucleotides, produces a global (non-specific) cDNA population from all transcripts in the RNA sample. The cDNA is then used for subsequent analysis in a qPCR step and interrogated for the sequences of interest using gene-specific PCR primers.

Common abbreviations

(q)PCR: (quantitative) polymerase chain reaction

A: Adenine

C: Cytosine

cDNA: complementary DNA

cfDNA: cell-free DNA

dNTP: deoxyribonucleotide triphosphate

dsDNA: double-stranded DNA

G: Guanine

gDNA: genomic DNA

GOI: Gene of Interest

miRNA: microRNA

mRNA: messenger RNA

ncRNA: non-coding RNA

RG: Reference Gene

rRNA: ribosomal RNA

RT-qPCR: Reverse Transcription qPCR

SDS: Sodium Dodecyl Sulfate (anionic detergent)

ssDNA: single-stranded DNA

T: Thymine

tRNA: transfer RNA

U: Uracyl

Section A. Technical information

A.1 PCR and qPCR

An understanding of the polymerase chain reaction (PCR) is required to explain the technique of real-time quantitative polymerase chain reaction (qPCR). PCR is an immensely powerful, genetic amplification technique resulting in high levels of sensitivity and specificity in bioanalysis.

PCR is an enzymatic reaction used to amplify DNA. DNA usually consists of a pair of complementary polynucleotide strands linked together to form a double helix. The strands are made up of the four bases: Adenine, Thymine, Guanine and Cytosine (abbreviated A, T, G, and C respectively) which pair with one other base, usually A to T, and C to G, on the complementary strand. When a DNA solution is heated, the non-covalent, hydrogen bonds that hold the two strands together weaken and eventually the two strands separate, denature or “melt”.

PCR enables targeted regions of small amounts of DNA to be exponentially amplified, generating larger amounts of the target region. For example, 500 copies of a DNA sequence may be amplified to 50 billion copies, which is much easier to measure using currently available technology. This reaction can be tailored in order to amplify specific sequences within the DNA, which are known as target sequences.

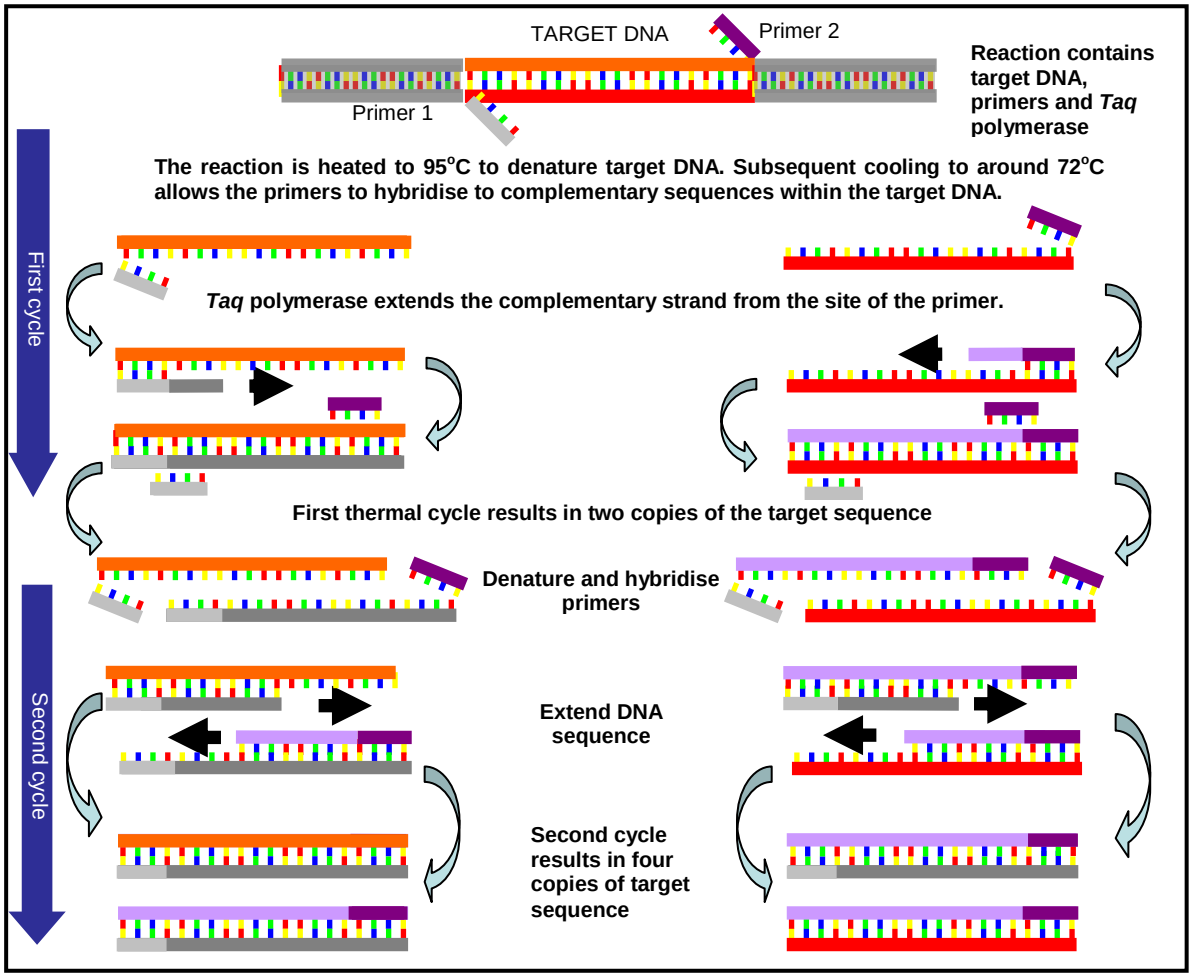


Figure A - 1 Schematic of the polymerase chain reaction. DNA duplex is separated in a high temperature denaturation step. Complementary primer sequences that flank the target sequences for amplification anneal to the target; this is termed the annealing step. Complementary nucleotides are polymerised into the growing strand by the DNA polymerase enzyme. Extension usually continues until the template sequence has been completely copied. The process is repeated, often between 25-40 times. Large numbers of copies of each original template are made via this exponential process.

The chosen target sequences are identified and “amplified” using primers. Primers are short oligonucleotides that are designed to recognise and flank the sequence of interest. Forward and reverse primers are designed to complement the antisense and sense DNA strand sequences, respectively. As with all enzymatic reactions, PCR necessitates optimal conditions which balance factors such as temperature, salt content and appropriate buffering, for the reaction to proceed efficiently (see qPCR assay design, section A.3).

In particular, PCR requires temperature cycling for the different stages of the reaction to occur (see Figure A - 1). Each stage requires a different temperature.

Stage 1: Denaturation of the dsDNA template – 94-95 °C typically for 1-30 seconds.

Stage 2: Annealing of sequence-specific primers to the ssDNA template – 50-65 °C typically for 1-40 seconds. The optimal annealing temperature of the primer is intrinsically linked to its sequence.

Stage 3: Extension of each primer in the 5’ to 3’ direction by DNA polymerase enzyme – 72 °C for elongation.

Detection of PCR products can be achieved using agarose gel electrophoresis or capillary electrophoresis with Lab-on-a-chip based platforms. This is known as end-point analysis or end-point PCR. Following PCR amplification, the amplicons generated are subjected to size separation and specific products may be identified based on the expected product size.

Unlike conventional PCR, which relies on end-point analysis, qPCR enables the analyst to monitor DNA amplification in real time, as the reaction proceeds. This allows accurate quantification of DNA targets. Quantitative approaches are based on the cycle at which amplification is first detected, rather than requiring quantification of PCR products. Usually, a range of samples of known target content is amplified together with the samples to be tested.

Real-time detection of the amplified DNA has evolved rapidly and normally involves the utilisation of fluorescent dye markers. A fluorescent marker is designed such that changes in its fluorescent output are in direct relationship to the reaction progress, i.e. how much DNA is synthesised. This in turn is coupled with PCR instruments developed for the purpose of detecting changes in fluorescence in real time. Expansion of the conventional PCR technique to include qPCR has streamlined analysis and exposed further potential applications. As a result, qPCR has been established as a key technique in molecular biology.

Within the research field, in addition to detection of DNA, qPCR is employed to provide quantitative measurements of gene expression. This technique, combined with the reverse transcription reaction (RT-qPCR) for mRNA conversion to complementary DNA (cDNA), is used to measure gene expression in response to a wide range of scenarios. Such applications include the administration of a pharmacological agent, progression of cell differentiation, changes in environmental conditions or disease evolution.

A.2 Sample purification and Quality Control (QC)

This section will describe specific quality considerations associated with the analysis of DNA and different types of RNA, such as determining the purity and degradation status of the material extracted from the sample. Possible approaches for quality control measures to ensure the validity of the qPCR analysis are also discussed.

A.2.1 Sample purification

Sample purification is the crucial first step for the vast majority of molecular biology techniques. The nucleic acid extracted from biological material needs to be sufficiently pure, concentrated and free of components which may inhibit enzymatic reactions. Quantitative measurements using qPCR are particularly susceptible to inhibition as small amounts of co-purified inhibitors can impact on the quantification (see Contaminants and inhibition, section A.2.3.2).

A.2.1.1 Sample purification – DNA

Purification is an essential step because DNA is frequently bound by proteins, contained within several cellular membranes and/or surrounded by extracellular matrices of different constituents.

Purification is usually achieved by an initial cell lysis step. Frequently chemical lysis is employed, however other lysis methods including enzymatic, thermal and physical steps for extraction may be utilised for complex samples which are difficult to purify (e.g. bone and gram negative bacteria). The lysis step can often be tailored to specific tissue and/or sample types. However, it is important to remember that extraction kits are often developed to work with commonly analysed sample types and may not always work as well with more obscure samples. Therefore, extraction from more challenging material may require some optimisation.

Once cell lysis has been achieved, the next step is the removal of the vast majority of the non-nucleic acid biological material, as well as any components from the chemical lysis that may be present. One of the many ways this may be achieved is by the combined use of phenol and chloroform, to bind and extract the protein constituents. After centrifugation the nucleic acid material remains in the aqueous phase, to be recovered by aspiration or precipitation. Phenol/chloroform extractions have been superseded by a plethora of kit-based methods from a variety of suppliers incorporating columns or magnetic beads, as well as the use of high throughput robotics.

Despite the choice available, the role of the extraction kit generally remains the same – to provide pure, concentrated, amplifiable nucleic acid. Kits may also be adapted to tailor the methods to specific nucleic acid types. Significant differences exist between DNA and RNA extraction methods. Emerging analytical requirements have resulted in methods that favour one over the other, such that it is actually difficult to identify a suitable method for the extraction of both RNA and DNA equally. However, this requirement is rarely necessary. In fact, the presence of DNA in RNA extractions is generally undesirable as DNA may contaminate the reverse-transcription PCR reaction, reducing efficiency of the RT and PCR steps and causing ambiguity over the identity of the (cDNA or gDNA) target amplified.

Further nucleic acid distinction may also be achieved by adapting current protocols. For example cell-free DNA (cfDNA) can be extracted from blood by removing the cell fraction and extracting nucleic acid from the remaining plasma or serum. Methods that enable increased focus on specific types of nucleic acids, e.g. micro RNAs (miRNA), are becoming more common when working with RNA, either as specific extraction methods or subsequent steps following total RNA extraction.

A.2.1.2 Sample purification – total RNA

The term “total RNA” has historically been used to describe extracts containing the traditionally defined RNA types: Ribosomal, Transfer and Messenger RNA (rRNA, tRNA and mRNA, respectively). However, the use of this term is now misleading as smaller non-coding RNAs (ncRNA) including microRNAs (miRNA) have become the object of increasing interest over the

last 10 years. Many of the classic extraction methods used for “total RNA” may not recover this fraction because these protocols are selective for larger molecules.

Notwithstanding the inaccuracy of the above definition, total RNA is the focus of research performed for gene expression analysis. The fraction of interest, the mRNA, constitutes only a small amount (2-5%) of the total RNA. The vast majority is rRNA (~80%) and it is this fraction that is generally used as the template for quality determination when performing RNA extractions (see Extract quality control, section A.2.2)

Historically, RNA has been considered to be more challenging to work with than DNA due to the instability conveyed by the additional oxygen molecule within its composition. In practice, and with the latest techniques, RNA stability is little more of an issue than DNA stability. The underlying issue with RNA based studies is due to the stability and ubiquity of the degrading enzyme, RNase A. There are currently many RNA extraction methods, either supplied as kits or using readily available RNase-free reagents which have reduced difficulties with handling RNA.

While it is correct to state that RNA (and DNA) degradation is less problematic than previously encountered, it must still be considered and monitored during the experimental procedure because the degradation state of the template can affect the final interpretation of the data, as discussed in Template degradation, section A.2.3.3.

A.2.1.3 Sample purification – Micro RNA (miRNA) and non-coding RNA (ncRNA)

Purification of smaller miRNAs and other ncRNA molecules offers additional challenges due to their smaller size. Many extraction methods rely on the fact that most RNA molecules behave differently to many of the contaminating solutes, and as a result, employ targeted column binding or precipitation. Some column purification steps are based on size exclusion, thus potentially excluding the very small molecules that are of interest.

Smaller RNA molecules tend to behave more like unwanted solutes, and therefore the steps to remove solutes need to be more stringent. It may also be desirable to extract smaller RNAs as a pure fraction, independent of the other total RNA molecules. For example, there are a number of kits that offer miRNA specific extractions or enrichments. While these kits are very practical, it is important that laboratories perform a validation experiment and compare several systems to ensure they are fit for the intended purpose.

A.2.2 Extract quality control

Tissue storage and treatment, as well as nucleic extraction procedures, are highly variable and therefore it is critical to define a reliable protocol for analysis of sample quality and quantity. The quantity of RNA is largely determined by the yield of rRNA, whereas the quality of RNA is determined by the purity (i.e. absence of potential inhibitors affecting downstream applications) and the degradation status (structural integrity) of the RNA molecules. However, there is a significant overlap between the two factors since it is difficult to assess the quality of a sample with low quantity of RNA, and parameters which affect RNA quality may interfere with the quantification process.

There are a number of semi-automated techniques that may be used to assess both RNA quantity and quality. It is worth acknowledging that the method chosen to measure the quality and quantity of RNA may also affect the final outcome of those measurements.[3,4]

A.2.2.1 Spectrophotometry based systems

Nucleic acids have traditionally been quantified by measuring UV absorption using a spectrophotometer. In its simplest form, the absorbance is measured at 260 nm (A_{260}) and 280 nm. The concentration of nucleic acid is determined using the Beer-Lambert law, which predicts a linear correlation between absorbance and concentration.

An A_{260} reading of 1.0 is equivalent to ~40 µg/mL of pure RNA and ~50 µg/mL of pure double-stranded DNA. The Optical Density (OD) at 260 nm is used to determine the RNA or DNA

concentration in a solution. However the correlation between concentration and absorbance only exists with OD readings up to approximately 2. The OD/concentration relationship relies upon the purity of the samples; evidently, contaminating substances with absorption at 260 nm or 280 nm will affect the estimated OD reading.

The presence of potentially contaminating substances may be crudely determined by measuring the A_{280} and A_{230} for proteins, and at A_{230} for chaotrophic salts and phenol. However it should be noted that the determination of protein contamination in DNA in this manner is not particularly sensitive.

Additionally, pH and the ionic strength of the buffer also disturb the OD. Significant variability in the A_{260}/A_{280} ratio has been shown when different sources of water are used to perform the spectrophotometric determinations. Variation in the pH of water (from 5.4 to 7.5-8.5) used for resuspending the RNA significantly increased RNA A_{260}/A_{280} ratios from approximately 1.5 to 2.0.[5] Since the OD reading is a measure of absorption, it cannot be used to evaluate sample quality and therefore this cannot be used as a test for sample integrity (see more in Appropriate controls, section A.2.4).

An example of a system utilising spectrophotometric analyses is the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific). It has a wide dynamic range of quantification and enables analysis of small samples.

With this instrument, surface tension is used to hold a column of liquid sample in place while a measurement is made. This is achieved by pipetting the sample (only 1-2 µL) directly onto a measurement pedestal. A measurement column is then drawn between the ends of two optical fibres to establish the measurement path. The measurement is typically obtained within 10 seconds.

A.2.2.2 Fluorescence based quality assessment systems

The use of fluorescent dyes to quantify nucleic acids has become a common alternative to absorbance spectrophotometry.[6,7,8] Fluorescent methods depend on the measurement of changes in the fluorescence characteristics of small molecules or dyes upon binding to, or intercalating with, nucleic acid. Although more expensive than absorbance spectrophotometry, fluorescence-based quantification is more sensitive, precise and often specific for the nucleic acid of interest.

Since fluorometers measure fluorescence in relative rather than absolute units, the measurement is first calibrated with a known concentration of a standard nucleic acid solution with characteristics similar to the sample to be measured. Following calibration, a single measurement can establish the concentration of nucleic acid in the solution, but typically a standard curve will be required to ascertain the linear range of the assay.

An example of a system that uses fluorescent analyses is the QuBit® 2.0 fluorometer (Life Technologies). This instrument can be used with a range of different fluorescence based quantification assays for the measurement of nucleic acid concentration in solution. The assays demonstrate a wide dynamic range for detection and are capable of accurately analysing small samples.

An overview of the common fluorescent dyes used to detect and quantify nucleic acids is given in Table A - 1, and specific details concerning their general properties are outlined in the text below.

Dye	Excitation /Emission (nm)	Specificity	Sensitivity	Advantages	Adverse performance influencing factors
DAPI	360/465	A-T	Unknown	Inexpensive	Detergents, polyphosphates
Hoechst 33258	360/465	A-T of dsDNA	10 ng/mL	Inexpensive Sensitive	pH, SDS
PicoGreen ®	485/535	All dsDNA	250 ng/mL	Sensitive	None demonstrated
RiboGreen ®	485/535	G of all nucleic acids	200 pg/mL	Quantification of RNA	Presence of any DNA
OliGreen®	485/535	T of all nucleic acids	100 pg/mL	Quantification of ssDNA Sensitive	Presence of DNA or RNA
Cyanine dyes (e.g. YOYO-1)	Various	dsDNA, some single-stranded oligonucleo-tides	0.5 to 2.5 ng/mL	Inexpensive Sensitive	Salt, ethanol, SDS, some variants show base sensitivity
Ethidium bromide	520/605	dsDNA and RNA	2 ng band, agarose gel	Inexpensive Sensitive	Degrades in presence of sodium nitrate and hypophosphorous acid. Must be stored in absence of light
SYBR® Green I	485/535	dsDNA	25 pg band, agarose gel	Inexpensive Non-mutagenic	Must be stored in absence of light

Table A - 1 Properties of common dyes used for the quantification of nucleic acids.

A.2.2.2.1 DAPI

The chemical 4',6-diamidino-2-phenylindole is generally referred to as DAPI. The compound exhibits a high specificity for DNA, and is categorised as a minor-groove binder. It forms a fluorescent complex only when bound to the minor groove of A-T rich sequences of DNA although it can form non-fluorescent intercalative complexes in other regions such as GC sequences or ssDNA. Although other minor-groove binding dyes (including the Hoechst dyes) exhibit a greater overall fluorescence yield compared to DAPI, it still demonstrates a near 20 fold increase in fluorescence when bound to dsDNA.

A.2.2.2.2 Hoechst dyes

These are a group of minor groove binding bisbenzimide compounds. They demonstrate specificity for A-T rich regions although they can also exhibit more complex DNA affinities. Hoechst dyes are generally excited by light in the near UV range (360 nm) and emit light in the blue range (460 nm). The most commonly selected dye for DNA detection and quantification is Hoechst dye 33258 which exhibits a strong selectivity for dsDNA.

A.2.2.2.3 PicoGreen®

PicoGreen® is a fluorescent dye developed and patented by Molecular Probes Inc. This dye exhibits a strong increase in fluorescence (>1000 times) when bound to dsDNA, but does not exhibit any significant increase of fluorescence in the presence of proteins, carbohydrates, ssDNA, RNA or free nucleotides. The dye also lacks specificity for A-T or G-C regions so that it may be used to bind DNA from any source. PicoGreen® has been shown to be capable of detecting DNA concentrations as low as 250 pg/mL, and to have an assay linearity which extends over four orders of magnitude.

A.2.2.2.4 RiboGreen®

RiboGreen® can be used to detect both RNA and DNA (ssDNA and dsDNA), as fluorescence of the reagent increases upon binding nucleic acids. By employing two dye concentrations, three orders of magnitude of sensitivity are possible, starting at a concentration of 1 ng/mL. RiboGreen® demonstrates some base selectivity, with a 60% decrease in fluorescence in the presence of poly (G) fragments and virtually no fluorescence in poly (U) or poly (C) fragments.

A.2.2.2.5 OliGreen®

OliGreen® is highly sensitive and is capable of detecting as little as 100 pg/mL of ssDNA. It does, however exhibit significant base selectivity for T, with little or no selectivity for A, C, or G. OliGreen® also exhibits significant increase in fluorescence in the presence of dsDNA and RNA.

A.2.2.2.6 Cyanine Dyes

This class of fluorochromes includes the TOTO and YOYO family of dyes (e.g. dimeric cyanine dyes), ethidium bromide and SYBR® Green I dye. Dimeric cyanine dyes may be used to quantify dsDNA, ssDNA and RNA. Cyanine dyes are not as sensitive as the PicoGreen® group of compounds as they only show a linear range of response over two orders of magnitude with a limit of sensitivity of 0.5 ng/mL. Applications of these dyes include:

- YOYO-1 for quantifying oligonucleotides and PCR products
- YO-PRO-1 for quantifying dsDNA in solution (reported sensitivity of 2.5 ng/mL)

Additional cyanine dyes and their applications include: ethidium bromide for quantifying DNA (in conjunction with agarose gel), and SYBR® Green I dye for quantifying nucleic acids. This dye is frequently employed in real-time PCR (qPCR) reactions for DNA quantification.

A.2.2.3 Gel electrophoresis and capillary systems

Capillary systems use a lab-on-a-chip approach, combining capillary electrophoresis with fluorescent detection. The electrophoretic process on the chip is based on traditional gel electrophoresis principles that have been miniaturised to a chip format. The chip format dramatically reduces sample consumption and separation time.

The chip accommodates sample wells, gel wells and a well for an external standard (fragment size ladder). During manufacturing, micro-channels are fabricated in glass to create interconnected networks amongst the wells. These micro-channels are then filled with a sieving polymer and fluorescence dye. Electrodes are inserted in the wells and the chip becomes an integrated electrical circuit.

Charged bio-molecules such as DNA or RNA are electrophoretically driven by a voltage gradient. Due to a constant mass-to-charge ratio and the presence of a sieving polymer matrix, the molecules are separated by size. Smaller fragments migrate faster than larger ones. Dye molecules intercalate into nucleic acid strands and these complexes are detected by laser-induced fluorescence. Data is then translated into gel-like images and electropherograms, exhibiting bands and peaks, respectively.

Depending on the size of the chip, a relatively small number of samples (10-12) can be processed.

A ladder standard is incorporated on every chip alongside the samples. This is used as a reference for data analysis. The software automatically compares the unknown samples to the ladder fragments to determine the concentration of the unknown samples and to identify the key features of the sample such as ribosomal RNA peaks or DNA fragments. In addition, these systems have various algorithms to provide an analysis of the quality of the sample.

An example of a capillary system is the Agilent 2100 Bioanalyzer. The Bioanalyzer uses two chips for the analysis of RNA: the Nano LabChip kit for the analysis of low amounts of RNA (25-500 ng/μL) and the Pico LabChip for even lower levels of RNA (50-5000 pg/μL). (Note that 50 pg of RNA is the equivalent to approximately 5 cells if 10 pg of total RNA per cell is assumed. For comparison, the amount of DNA per cell is approximately 7 pg.) The Agilent system employs an algorithm that interrogates the mobility of all fractions of RNA and which produces a RNA Integrity Number (RIN). It is generally regarded that a sample with a RIN of 10 is indicative of the best quality RNA whereas lower values result from RNA degradation.

A.2.3 Contaminants and inhibition

Sample quality is critical and therefore it must be verified before the samples are used in qPCR assays. It has been clearly demonstrated that analysis of degraded RNA samples can result in poor quality data [4]. In addition it has been shown that the presence of inhibitors differentially affects qPCR assays.[9] Therefore it is important to determine the integrity and purity of nucleic acid samples.

A.2.3.1 Contamination

In general terms, contamination refers to the presence of an unwanted component in a sample.

Classic examples of contamination in biological research experiments include the presence of mycoplasma in cell culture or unwanted proteases during serological analysis.

Within the context of qPCR assays, the significant forms of contamination refer to nucleic acid template present in the sample which may be detected along with the specific target, thus generating a false positive, or material that may inhibit downstream reactions resulting in a reduced or failed reaction (false negative).

A.2.3.1.1 Amplicon (target sequence) contamination

PCR assays are especially vulnerable to contamination with the specific target sequence of interest for two reasons:

a) PCR is a process that generates billions of copies of the amplicon, the specific target of interest. However, amplified product from one PCR is potentially a source of contamination for future PCRs. In a molecular biology laboratory, it is therefore essential to separate the PCR set up area (the designated space in which the reaction is prepared) from the post-PCR analysis area (the site the product will be subsequently analysed and possibly manipulated). This should be achieved by using separate rooms with dedicated equipment and laboratory coats such that nothing from the post-PCR analysis space is brought into contact with the clean (pre-PCR) space. Many laboratories also introduce a one way policy such that if an individual has entered a post-PCR room they are forbidden from subsequently entering a pre-PCR room on the same day.
b) qPCR assays are particularly vulnerable to amplicon contamination given the scope of detecting a single template molecule; hence minuscule amounts of contamination are sufficient to cause a problem.

A.2.3.1.2 Other sources of template contamination

In addition to PCR generation of the specific amplicon, care is needed in the laboratory setup to ensure that template material is not transferred from one sample to another (i.e. cross-contamination). In the case of analysis of human samples, there must be precautions to ensure that material is not introduced from the analyst.

It is important to identify and avoid contamination of all samples, and the use of controls for this purpose is highly recommended (see section A.2.4, Appropriate controls).

A.2.3.1.3 Contamination of RNA with gDNA

When analysing RNA, it is important to ensure there is no genomic DNA contamination (gDNA), especially if the assays in use do not discriminate between the gDNA and cDNA sequences. Enzymatic treatment of the samples with DNase I is recommended to remove contaminating gDNA, and is particularly important for investigations of intronless genes. Although a column-based DNase I digestion step is more practical, a post-extraction solution phase DNase treatment may remove the gDNA more effectively.

Wherever possible, it is advisable to design assays over the intron/exon boundary such that only the processed mRNA sequences can be amplified and/or detected. This does not always prevent amplification from gDNA, as there are sequences in existence as processed pseudo genes which are effectively genomic cDNA sequences. It is also worth noting that template DNA often exists in commercially available reagents which can cause issues.[10]

A.2.3.2 Inhibition

The presence of residual inhibitors in a sample may result in complex perturbations of the true data. Distinct inhibitors exhibit differential effects on the assay, leading to incorrect estimates of relative target quantities, or difficulties in interpreting genotyping assays.

Several potential results may be recorded when measuring transcript quantities in samples containing inhibitors. Since the adverse effect on each assay is inherently different, the resulting ratios between GOI quantities and reference genes may not reflect the true abundance of each gene.

Common inhibitors that are experimentally introduced include: tris, ethanol, isopropanol, EDTA, the reverse transcriptase enzyme, guanidine isothiocyanite and phenol.

One system that can be used to detect inhibitors is referred to as the SPUD assay.[11] Using this method, an artificial amplicon (derived from a potato nucleic acid sequence lacking homology with any other known animal or human sequence) is subjected to qPCR in the presence of either water (amplification control) or the test sample. In the absence of any SPUD assay inhibitors in the test sample, the quantification cycle (C_q) recorded for the amplification control and test sample will be identical. However, if the test sample contains an inhibitor, the C_q will be increased (see Figure A - 2 and also SPUD protocol, section C.2).

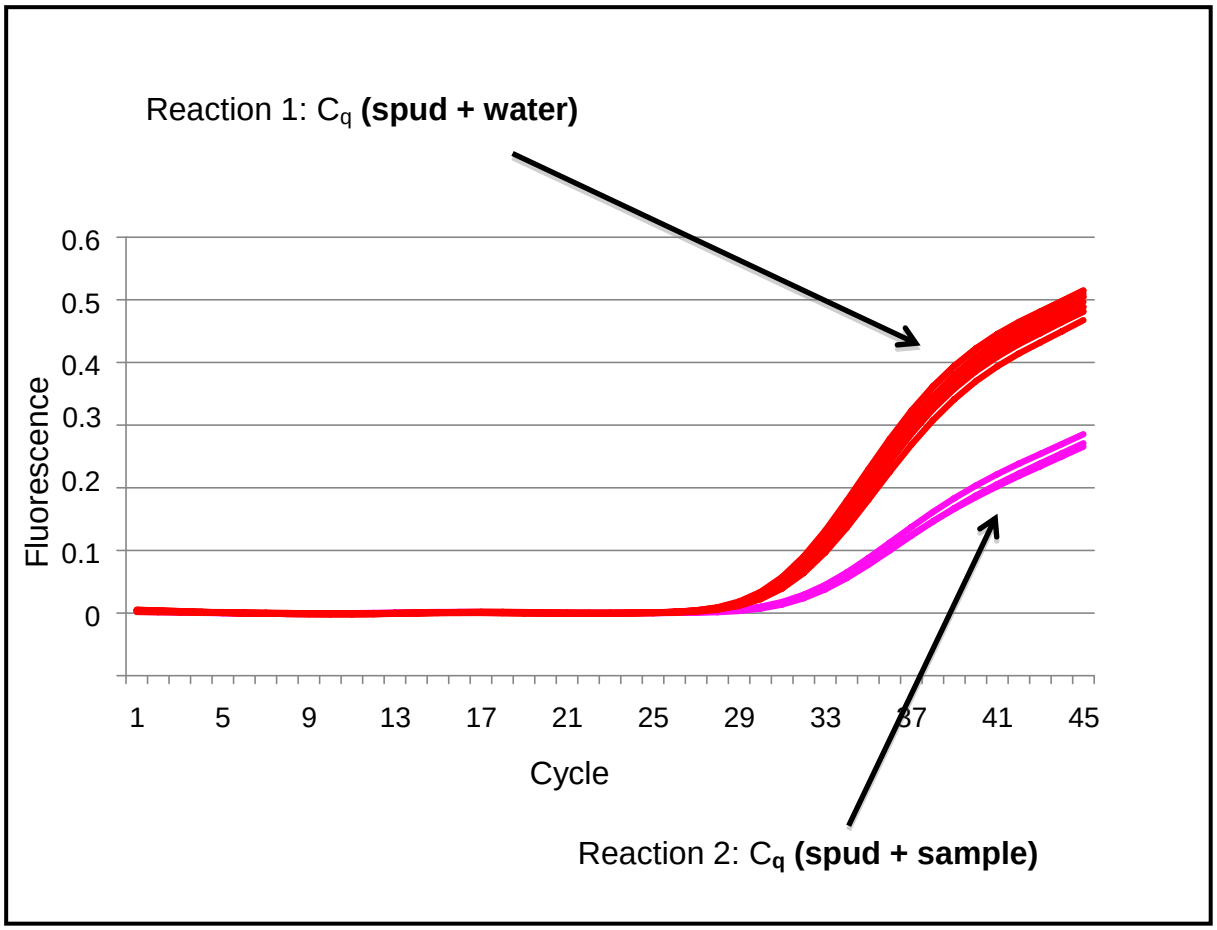


Figure A - 2 Representation of the SPUD assay. In this case, the difference in C_q for the sample and the control shows presence of an inhibitor.

A.2.3.3 Template degradation (3'/5')

It has been demonstrated that individual transcripts in a total RNA sample are subject to differential degradation[12] and that the status of the rRNA is not a reliable indicator of the integrity of the mRNA. Therefore, it is important to determine the degradation state of the RNA samples. Some indication can be derived from electrophoretic analysis using an agarose gel or bioanalyzer analysis.

However, it has been observed that samples exposed on bare human skin for 1 minute, exhibited no apparent degradation when the total RNA was subsequently analysed using an Agilent Bioanalyzer. [13] For this reason it is important to apply a transcript-specific investigation. One such approach relies on measurement of a target using two independent assays. The integrity assay relies on measuring the ratio of the quantities of the target located at the 5' and target located at the 3' of the same transcript. This provides a relative measure of RNA integrity (for a 3'/5' assay protocol, see section C.3).

In this way, the 3'/5' ratio assay is used to evaluate the quality of RNA samples. The approach is demonstrated in section C.3, by testing the state of the GAPDH transcript; the specific targets under examination should be tested in any given experiment. After an oligo-dT primed cDNA synthesis, the GAPDH transcript is quantified using both a 5' and 3' assay. If the RNA is intact an equal concentration is expected using each assay (ratio = 1), whereas a higher copy number of the 3' assay relative to the 5' is expected if the RNA is degraded.

A.2.4 Appropriate controls

As with any technique, it is essential that qPCR experiments have correct controls. Appropriate negative or no template controls (NTC) are essential for detecting contamination of the PCR assay, and unintended amplification products such as amplified primer-dimers which may occur with the use of dsDNA binding dyes. The nature of the control must accurately reflect the test samples. For example, if the aim is to detect a pathogen in human stool, the NTC cannot simply be made with water as the template replacement; it must be prepared using a stool sample that is known not to harbour the pathogen.

Additionally, it may be appropriate to include different types of negative control samples, which provide quality control information regarding experimental procedures prior to qPCR. For example, nucleic acid extraction blanks and reverse transcription negatives obtained by omitting RT enzyme. Negative controls should be included alongside test samples, with both PCR set-up controls and negative controls having been subjected to the same extraction and preparation processes as the samples analysed. It is advisable to incorporate a number of negative controls and intersperse their preparation with that of the test samples to obtain a representative estimation of the level of contamination in the analytical process. Without the inclusion of such controls it may be impossible to determine whether signals arise from the amplification of endogenous sample targets, or whether cross-contamination between samples has occurred.

Positive controls also provide valuable quality assurance and are essential when measuring low copy numbers or where a negative result needs to be assigned with confidence. Dilution curves are often sufficient for this purpose, but other positive controls may be included. The positive controls are used to qualify the functionality of the reaction components and to assess the efficiency of the assay. Internal positive control reactions can also be used to demonstrate that no reaction inhibition has occurred,[14] which is particularly important in the interpretation of apparently negative results from clinical or environmental samples. Inclusion of characterised positive samples may also be used to compare with test samples in post-amplification melt curve analysis (see Melt curve analysis, section A.3.5.2) and these are useful for assessing reaction specificity for genotyping assays.

A.2.5 Reverse transcription

Reverse transcription (RT) is the process of converting RNA to cDNA, using a reverse transcription enzyme and dNTPs, prior to analysis of the cDNA in the qPCR step.

The RT step may be performed on total RNA such that a global cDNA representation of many transcripts is produced (usually via a two-step protocol). In a gene-specific approach, only the RNA of interest is converted to cDNA (usually following a one-step protocol).

Since it has been demonstrated that the two-step RT reaction does not always exhibit linearity with respect to input RNA and cDNA yield,[15] it is also important to determine and control the total amount of RNA extracted and incorporated in RT reactions. The relative concentration of RNA influences the efficiency of the RT and the concentration of cDNA produced from a given transcript. For this reason, it is desirable to include the same or a very similar concentration of RNA in all two-step cDNA synthesis reactions unless the RT system has been verified to exhibit a linear response.

A.2.5.1 Reverse transcription priming for two-step RT reactions

There are broadly two approaches used for two-step RT priming. The initial method relies on an oligo-dT (usually 15mer) to prime the poly A tail present on the majority of mRNAs, and thus selectively reverse-transcribe mRNA.

The approach, while conceptually very simple, conveys a number of problems; at temperatures used to perform the RT, the oligo-dT will bind non-specifically to other regions of RNA. Additionally, stretches of rRNAs are also detected as AT rich regions in these molecules are

primed by oligo-dT. Some mRNAs, such as those encoding histones, which do not contain poly A tails will not be represented within the resultant cDNA.

Despite the limitations, the potential for oligo-dT priming to target the 3' end of the mRNA has made this approach useful for studies investigating whole RNA molecules. This approach is also currently employed in some next generation sequencing methods for RNA analysis.

The second method uses random priming. Random primers consist of random sequences, frequently hexamers (6-mer) or nonamers (9-mer). They are used in the reaction to produce cDNA fragments of varying length which represent the original RNA. Random primers hybridise along the length of the transcript and tend to be more tolerant of secondary structure. To benefit from the advantages of the respective techniques some protocols necessitate a combination of both primer types. A specific primer to the target sequence may also be used in a two-step RT protocol (see below).

A.2.5.2 Reverse transcription priming for one-step RT reaction

In a one-step protocol, gene-specific primers are used to reverse transcribe a single target. The design of the gene-specific primer is critical; it must ideally reside within an open region of the mRNA target predicted (using predictive tools described in Tools for assay design, section A.3.4) to be accessible at the temperature of the RT reaction. Under these conditions there is a linear relationship between input RNA and cDNA.

Reverse transcription is a highly variable process and all steps must be considered to keep reaction components as constant as possible. Performing a one-step RT-qPCR reaction reduces the number of pipetting steps required, thus reducing error. It may therefore be the method of choice if small differences need to be measured and accuracy is paramount.

A.2.5.3 Reverse transcription efficiency

It is generally assumed that all the RNA/mRNA in a RT reaction is converted to cDNA. However, this assumption is incorrect. It is also assumed that all transcripts are converted in a 1:1 ratio or proportionally to the starting RNA concentration. Recent studies have been completed to investigate each of these assumptions. It is clear that that the amount of RNA converted to cDNA is highly variable.[16] The two-step RT process is variable and specifically dependent on RNA concentration, enzyme, buffer composition and priming protocol. Since the process is so variable, it is important to maintain as many constant conditions as possible.

For two-step RT reactions, it is necessary to aim for the same input RNA concentration, maintain constant priming conditions, RT enzyme and buffer components. When a constant input concentration cannot be determined it is advisable to employ a one-step process, and include a carrier such as Polyethylene Glycol (PEG) to enhance reaction performance,[17] or select a commercial kit that has been validated to result in a linear response.

A.3 qPCR assay design

Several factors should be considered when designing any experiment. These factors include selection of appropriate type and number of samples, the process stages for replication, the controls that should be incorporated, the need for randomisation of sample and standard arrangement within the experiment. In the case of qPCR assays, as well as these standard considerations, there is also a requirement to design adequate oligonucleotides and select appropriate detection chemistries. This section includes discussion of these factors and available options.

A.3.1 Replicates and randomisation

Performing sufficient replicate measurements of an unknown sample can increase the confidence with which the quantitative data is interpreted, and is often crucial in providing sufficient analytical information when measuring low concentrations of nucleic acid.

For quantitative determinations, it is generally accepted that a minimum of six replicates is required to obtain reasonable confidence in a result.[18] However, constraints on sample availability, time or cost may necessitate some reduction from this ideal. Another consideration is the stage at which to replicate the experiment (see Figure A - 3 for a graphical illustration of different replication strategies). For a typical qPCR experiment, replicates can be made at the:

- Biological level (different but experimentally equivalent samples, e.g. multiple animals from a control and a test group);
- Nucleic acid extraction stage (where the same sample is used to produce multiple nucleic acid samples);
- RT step (multiple cDNA samples are generated from a single RNA sample);
- PCR stage (same extract measured multiple times).

Decisions regarding the level at which replication should occur will depend on the aims of the experiment. For example, when trying to estimate Genetically Modified (GM) ingredients in a foodstuff, extraction replicates will be important. However, for a clinical study, biological replicates may be more informative. In general, for biological measurement (as opposed to experiments evaluating the extraction technique or PCR reaction specifically), replicates should primarily be at the biological level. Many laboratories no longer replicate the PCR reaction at all (technical replicate), in favour of increasing the number of biological replicates.

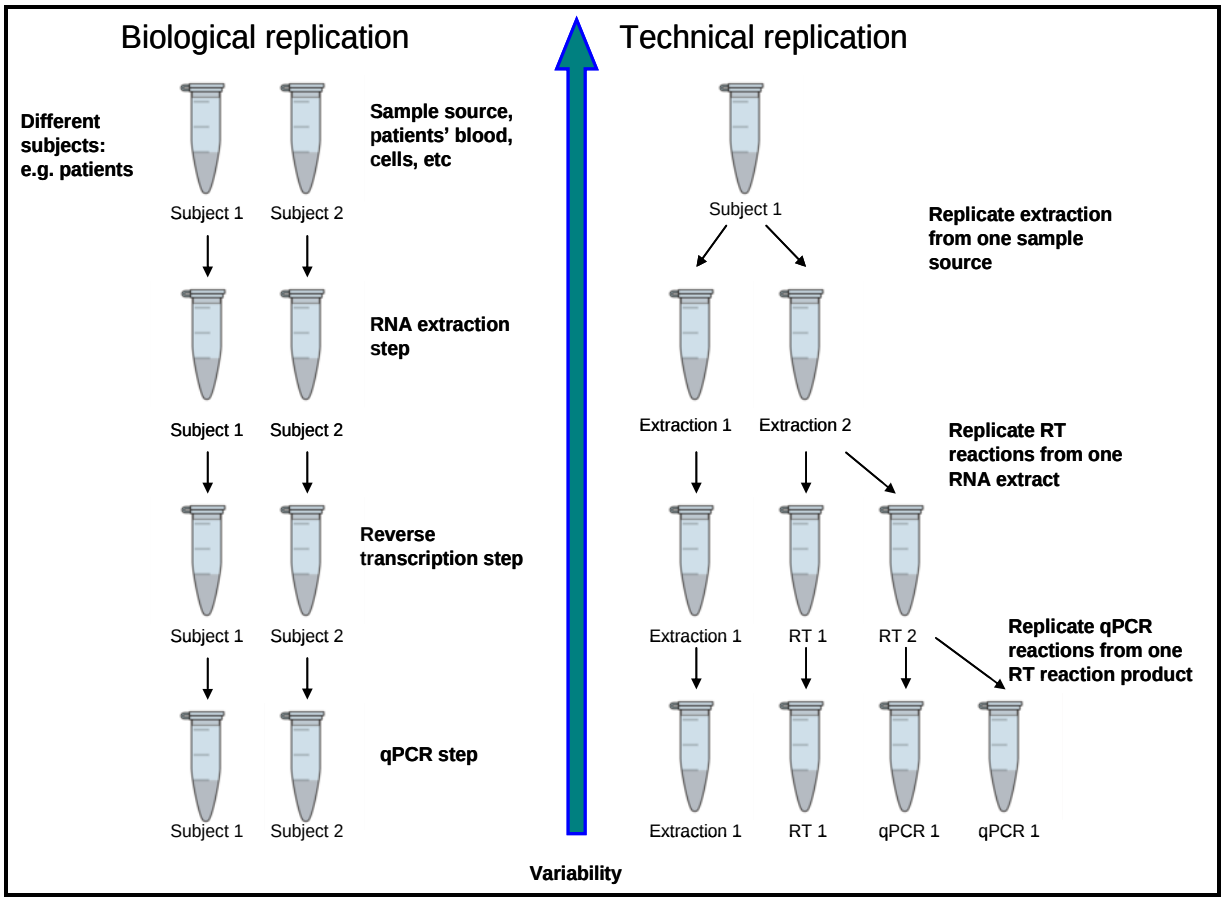


Figure A - 3 Schematic representation of different experimental designs illustrating biological versus technical replication. Data variability increases as replication is included from further back in the process. For example, to see true patient variability, replicate biological samples must be analysed (different samples from one patient, samples from different tissues from the same patient, or samples from different patients). The RNA extraction and reverse transcription components of the process may contribute more variability to the final measurement than qPCR *per se*. This increased variability is more representative of the actual variability and as such, more confidence can be conferred to the results when this variability is included.

Depending on the aim of the experiment, it may be desirable to randomise the arrangement of samples, standards and controls on the qPCR plate (or rotor position in the qPCR instrument) to avoid any amplification bias resulting from temperature variations between the reaction positions. This is of greater relevance, for example, when assessing sample homogeneity and/or stability. However, while randomisation is not routinely used there is increasing evidence that it is an important consideration.[19]

A.3.2 Selecting the amplicon

The amplicon is the target sequence to be analysed. The following should be considered when selecting the amplicon:

- Aim for a qPCR amplicon length of 75-150 bases;
- Test the target sequence using mfold (http://mfold.rna.albany.edu/) modelling at the annealing temperature and aim for open structures (particularly important for the RT primer site);
- Avoid stem loop secondary structures with low -ΔG values (i.e. make sure the amplicon is within a structurally stable section);
- Avoid palindromic sequences;

- Avoid G:C rich areas and aim for approximately 50% G:C content;
- Avoid repetitive regions;
- Avoid regions with SNPs. A single mismatch between the primer and the template can decrease the melting temperature by up to 10 °C, affecting the efficiency of PCR;
- For transcript specific designs (to avoid detection of gDNA templates) target regions over the intron-exon boundary.

A.3.3 Primer and probe design

For most applications, primers are designed to be fully complementary to the template DNA sequences they are intended to prime. The basic considerations are similar to those for the design of conventional PCR primers:

- Typically, primers should be designed to range from 20 to 24 nucleotides in length, with a melting temperature (T_m) of approximately 60 °C (59±2 °C);
- Primer pairs should possess 40-60% G:C content and should lack significant secondary structures;
- It is important to avoid regions complementary to themselves or their partner, a fact that is especially important at the 3’ end to reduce the potential for the formation of primer-dimer products during amplification;
- The probe length is typically 20-30 bases long (except for Scorpions which have a probe of 15-25 bases long);
- Additionally, when using hydrolysis probes the T_m should be 7 to 10 °C higher than that of the primers to ensure that the probe has bound before the primers hybridise and extension begins;
- For quantitative studies using hydrolysis probes, aim for the probe to be sited close to the 3’ of the forward primer but not overlapping (around five bases). For SNP detection, position the probe in the centre of the amplicon;
- Avoid a guanidine at the 5' end of probes, next to the reporter, since this causes quenching;
- Ensure that there are no more Gs than Cs in the probe sequence.

Of course these are the ideals; the target sequence dictates what the primer sequences will be. It may not always be possible to achieve these criteria, which is why assay-specific optimisation is crucial.

It is also possible to introduce modified nucleotides, such as locked nucleic acid (LNA). A LNA is a modified RNA nucleotide. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon (see Figure A - 4). The bridge "locks" the ribose in the 3'-endo conformation. LNA nucleotides can be mixed with DNA or RNA residues in the oligonucleotide whenever desired. Such oligomers are synthesised chemically and are commercially available.

The LNA modification results in increased thermal stability, accommodating the design of shorter probes. These are more specific than oligonucleotides comprised of DNA alone and ideally suited to SNP detection. Additional applications of LNA modifications are for the design of oligonucleotides for analysis of difficult sequences, such as viruses, where a high degree of variability can make it difficult to design a generic assay.

The LNA modification also provides protection against nuclease digestion making these oligonucleotides suitable for *in vivo* use.

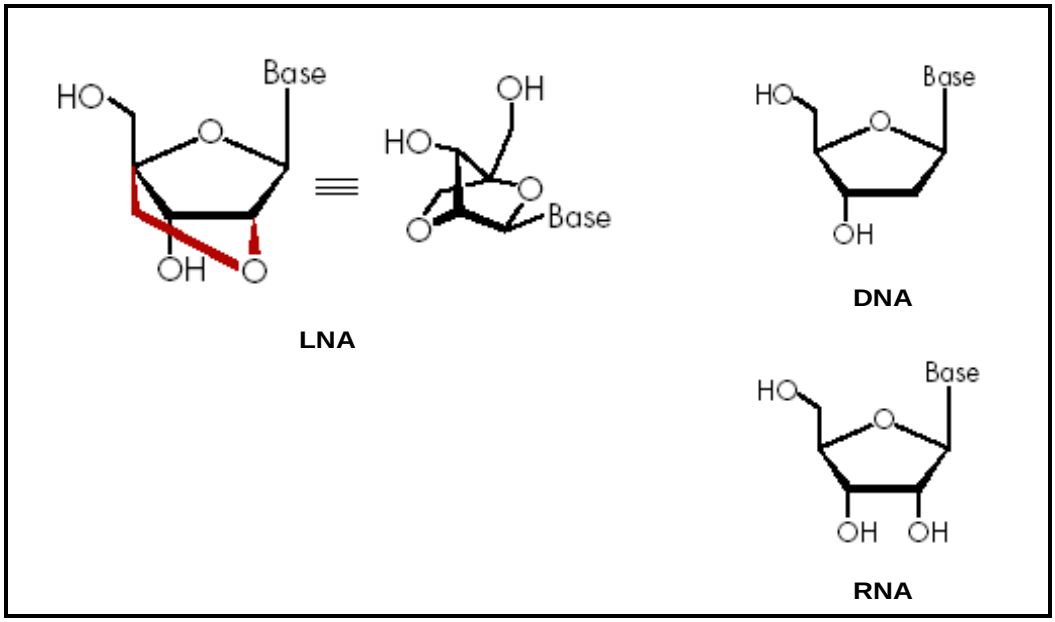


Figure A - 4 Configuration of a Locked Nucleic Acid compared to DNA and RNA. In LNA, the furanose ring conformation is restricted by a methylene bridge that connects the 2'-O position to the 4'-O position.

A.3.4 Tools for assay design

Assay design and validation is a worthwhile investment of time and effort. To assist with assay design, a range of validated assays are available online from the RT Primer database [http://www.rtprimerdb.org/].

Alternatively, there are several complementary design services provided by oligonucleotide manufacturers that support the design of a custom assay and will provide all the information about the assay design:

- NCBI GenBank: http://www.ncbi.nlm.nih.gov/genbank/
- OligoArchitect: http://www.sigmaaldrich.com/life-science/custom-oligos/dna-probes/product-lines/probe-design-services.html
- Beacon Designer: http://www.premierbiosoft.com/qpcr/index.html (provide a software package for purchase)
- Primer3: http://frodo.wi.mit.edu/primer3/
- PrimerBank: http://pga.mgh.harvard.edu/primerbank/
- NCBI Primer Design Tool: http://www.ncbi.nlm.nih.gov/tools/primer-blast/
- Mfold Webserver: http://mfold.rna.albany.edu/
- Although not a design package, RTPrimerDB is a database of pre-designed and validated qPCR assays http://medgen.ugent.be/rtprimerdb/

A.3.5 Detection chemistries

In order to monitor the change in DNA concentration as the qPCR progresses, fluorescent dyes or probes are included in the reaction mix. There are a number of options available which are discussed in the following sections.

A.3.5.1 Double-stranded DNA binding dyes

The group of dyes referred to as double-stranded DNA (dsDNA) binding dyes are frequently used in qPCR. As the DNA template is amplified during the course of the reaction, the amount of dsDNA increases, thus resulting in an increase in the number of dye molecules that bind to the amplicons (see Figure A - 5). Typically, when these dyes are free in solution, they absorb energy after excitation. This energy is then dispersed as heat (by increasing their molecular movement). However, when the dye is bound to dsDNA, its movement is impeded and this energy is dispersed as light (fluorescence) at a longer wavelength to that used for excitation. This fluorescence is measured at each cycle and is recorded over time; the signal increases in proportion to the increase in DNA concentration. These dyes are also used for post-amplification melt curve analysis, which enable additional examination of the amplified molecule (see Melt curve analysis, Section A.3.5.2). At the time of writing, SYBR® Green I dye is the most commonly used double-stranded DNA dye for qPCR. More recently a new generation of dsDNA binding dyes, including EvaGreen®, BEBO and BOXTO has been developed. These dyes have reduced PCR inhibition, have a lower background signal, and are also able to incorporate more dye molecules, therefore providing better measurement of amplification and enabling improved or "high resolution" melt analysis (see Terminology, page 3).

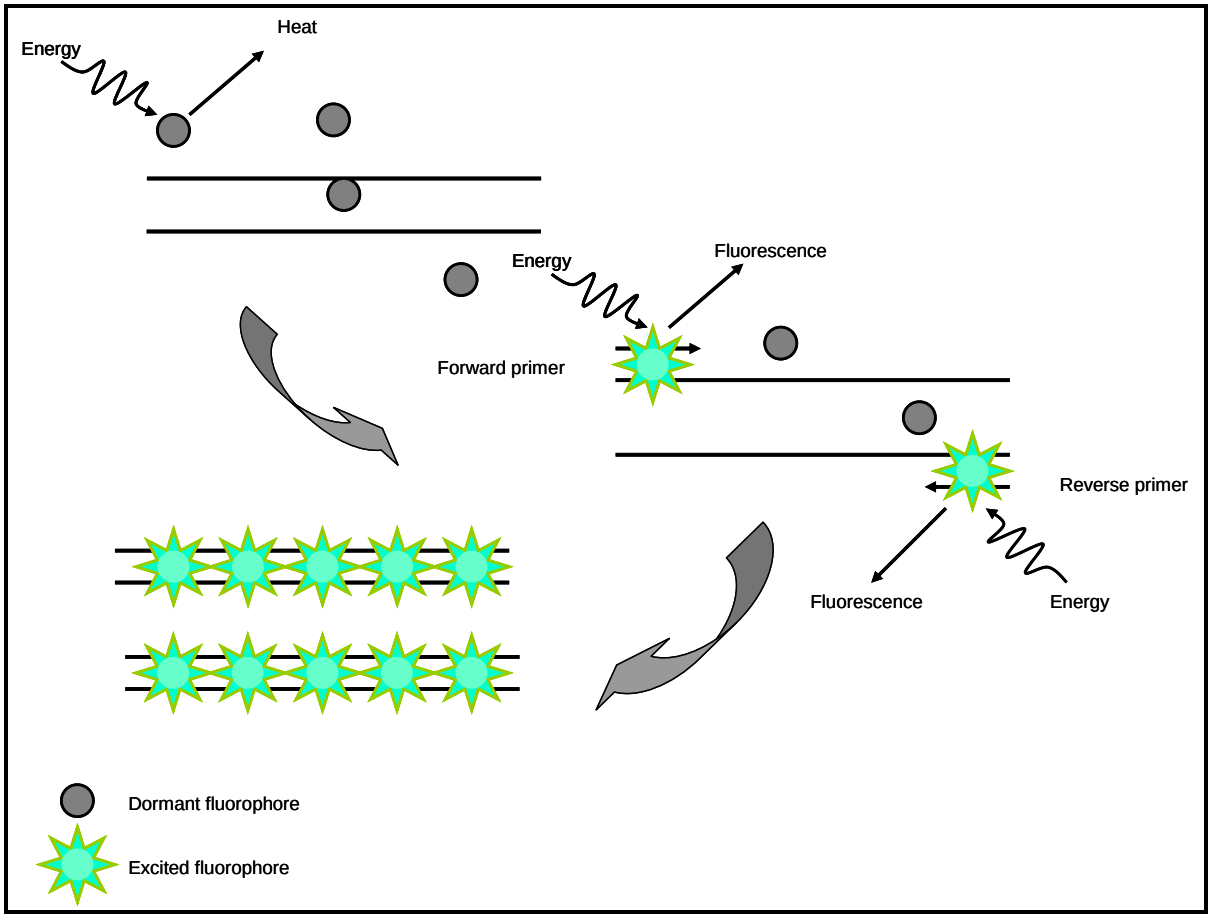


Figure A - 5 Schematic of dsDNA dye detection method. Energy absorbed by a dormant fluorophore that is free in solution is emitted as heat. Once a fluorophore molecule binds to dsDNA, any energy absorbed is emitted as light (fluorescence) at a specific wavelength. Binding to dsDNA is non-specific. The concentration of dsDNA increases as PCR progresses and this correlates to the increase in fluorescence.

A.3.5.2 Melt curve analysis

Melt curve analysis involves subjecting double-stranded PCR products to a gradual increase in temperature. The dsDNA begins to dissociate as the temperature is increased and results in a sharp decrease in fluorescence as the probe can no longer bind to the PCR product.

Theoretically, when 50% of the PCR product has dissociated, the T_m of that PCR product has been reached. Such melting temperatures may be PCR product specific, and is dependent upon the length of the product and the type of nucleotides present. This has allowed differentiation between the target specific amplicon and any non-specific amplicons as the different PCR products often have different bases present. Melt curve analysis can often be implemented following qPCR on the same instrument, improving the discriminatory power of the assay.

A.3.5.3 Target-specific probes

Alternative detection systems to dsDNA binding dyes are the family of fluorescent probes. Unlike dsDNA dyes, these probes only bind to defined target DNA, providing an additional level of specificity. The most commonly used probe types are hydrolysis probes (known also as TaqMan® or dual-labelled hydrolysis probes).

Hydrolysis probes are sequence-specific oligonucleotides designed to bind to the target sequence in between the PCR primers. The probe oligonucleotide is modified such that it contains a fluorescent reporter at the 5' end and a quencher at the 3' end (see Figure A - 6). When the probe is intact, the fluorescent reporter and quencher are in close proximity and as a result the fluorescent signal is quenched through fluorescence resonance energy transfer (FRET). The quencher absorbs fluorescence emitted from the reporter, typically dispersing it as longer wavelength light or as heat. The probe binds its complementary sequence within the target DNA in the same way as the primers. As elongation proceeds, the DNA polymerase extends the primer in the 5'-3' direction. When the DNA polymerase reaches the probe, the 5'-3' exonuclease activity of the polymerase cleaves the probe, liberating the 5' fluorescent reporter from the 3' quencher. As a result, the fluorescent reporter is no longer quenched and will emit fluorescence when excited. As the target is amplified, more reporter molecules are separated from their quencher, and so the fluorescent signal increases exponentially, in proportion to the amplicon concentration. This change in fluorescence with respect to PCR cycle may be used to quantify the relative amount of target DNA present in the sample when referred to a series of samples of known concentration which are used to prepare a standard curve (see Standard curve, section A.4.5.1.1). As the probes are sequence-specific, the fluorescence will only increase as a result of amplification of the target DNA sequence.

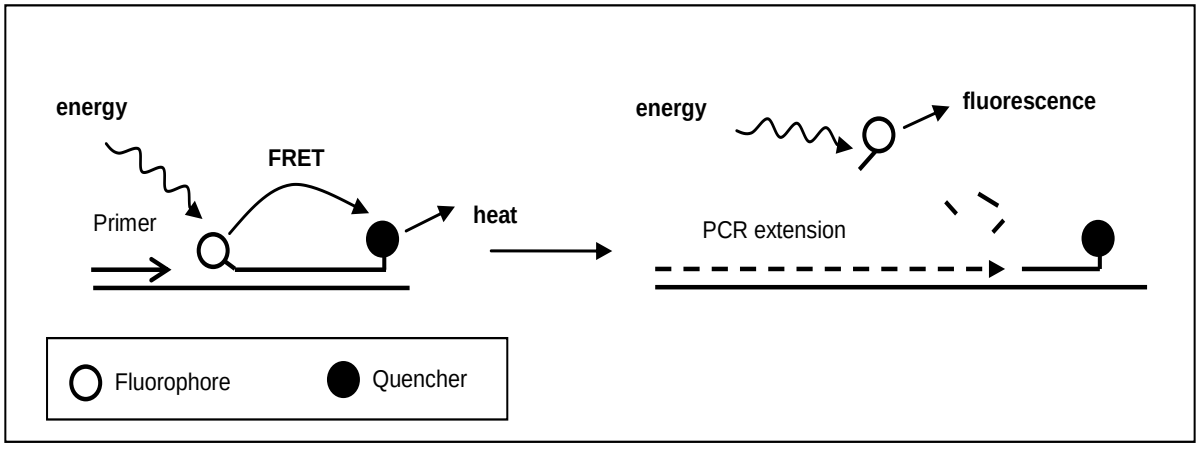


Figure A - 6 Illustration of the basis of the hydrolysis probe assay (reproduced from "Essentials of Nucleic Acid Analysis", RSC publishing, page 134).

Fluorescent probes may also be used in multiplex reactions, where several different targets are amplified simultaneously. This is performed using probes that have been labelled with fluorescent reporters possessing distinct excitation and emission profiles, allowing different targets to be monitored during the same PCR reaction.

A.3.5.4 Molecular beacons

Molecular beacons are single-stranded oligonucleotide hybridisation probes that form a stem-and-loop, or hairpin, structure in solution (Figure A - 7). The loop contains a probe sequence that is complementary to the target sequence, and the stem is formed by the annealing of complementary arm sequences located on either side of the probe sequence. A fluorophore is covalently linked to the 5' end of one arm and a quencher is covalently attached to the 3' end of the other arm. Molecular beacons have low background fluorescence when free in solution. However, when they hybridise to a nucleic acid strand containing a target sequence, they undergo a conformational change that causes the stems to open, thus separating the fluorophore and quencher and resulting in fluorescence.

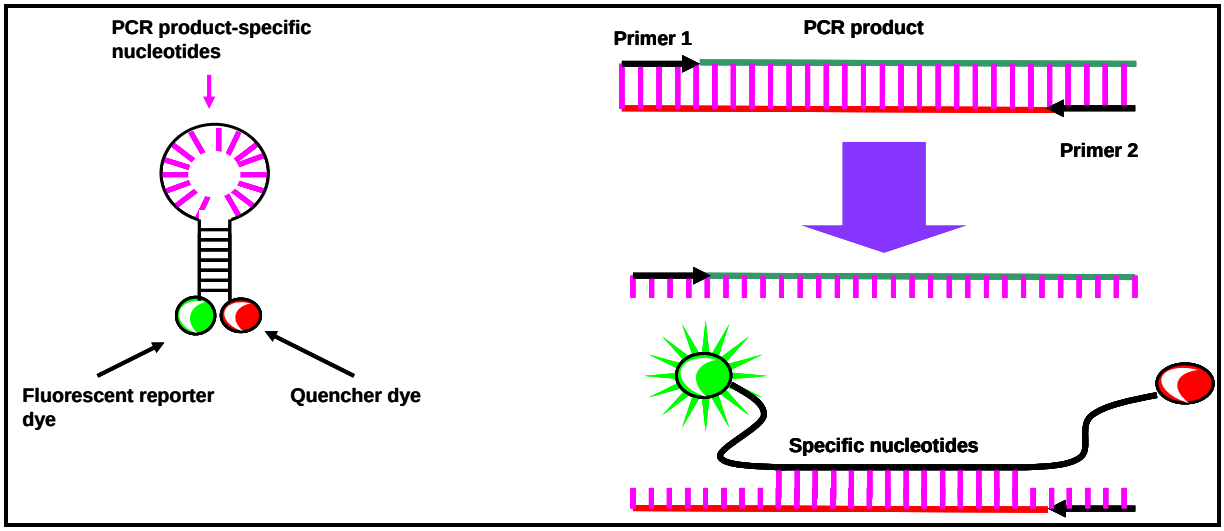


Figure A - 7 An illustration of molecular beacons. Prior to hybridisation the molecular beacon closed loop structure causes the fluorescent reporter and quencher to be in close proximity and therefore results in low fluorescence. During hybridisation the stem opens, physically separating the fluorescent reporter and the quencher and causing an increase in fluorescence.

A.3.5.5 Scorpions®

Scorpion® probes resemble the stem loop structure of the molecular beacon. However, the molecule also incorporates a covalently linked forward primer. In the inactive form the fluorophore attached at the 5' end forms a non-fluorescent complex with the quencher at the 3' end of the stem. The hairpin loop is linked to the 5' end of a specific primer through a PCR stopper (hexaethyleneglycol, HEG) that prevents read-through of the hairpin loop by the DNA polymerase. The qPCR reaction contains the Scorpion® probe and a single reverse primer (see Figure A - 8).

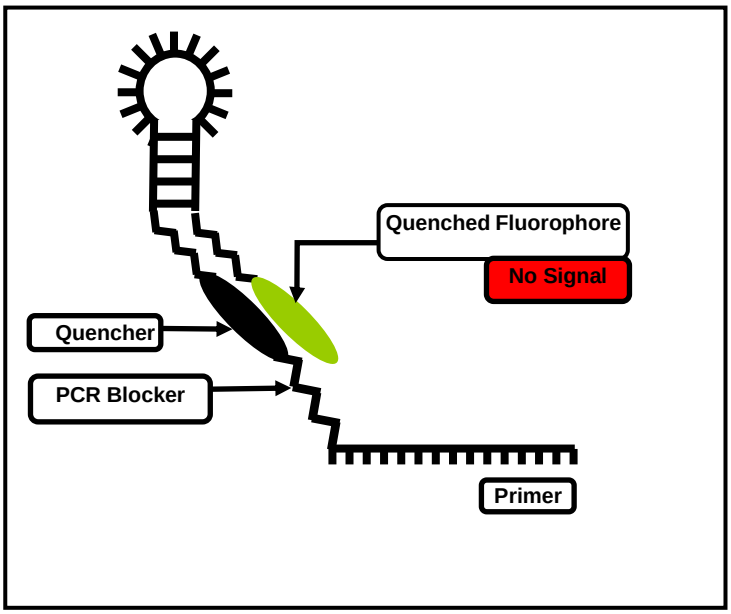


Figure A - 8 Structure of a Scorpion® probe.

During qPCR, the Scorpion® primers are extended and so become part of the newly formed amplicon. During the subsequent annealing/extension phase of the qPCR, the probe sequence in the Scorpion® hybridises to the newly formed complementary target sequence in the qPCR product. This results in separation of the fluorophore from the quencher and a subsequent fluorescent signal. As the Scorpion® and the PCR product are now part of the same strand of DNA, the detection interaction is intramolecular. Intramolecular detection is a much more rapid reaction than alternative probe detection systems. The target sequence is typically chosen to be between 5 and 50 bases of the 3' end of the Scorpion primer.

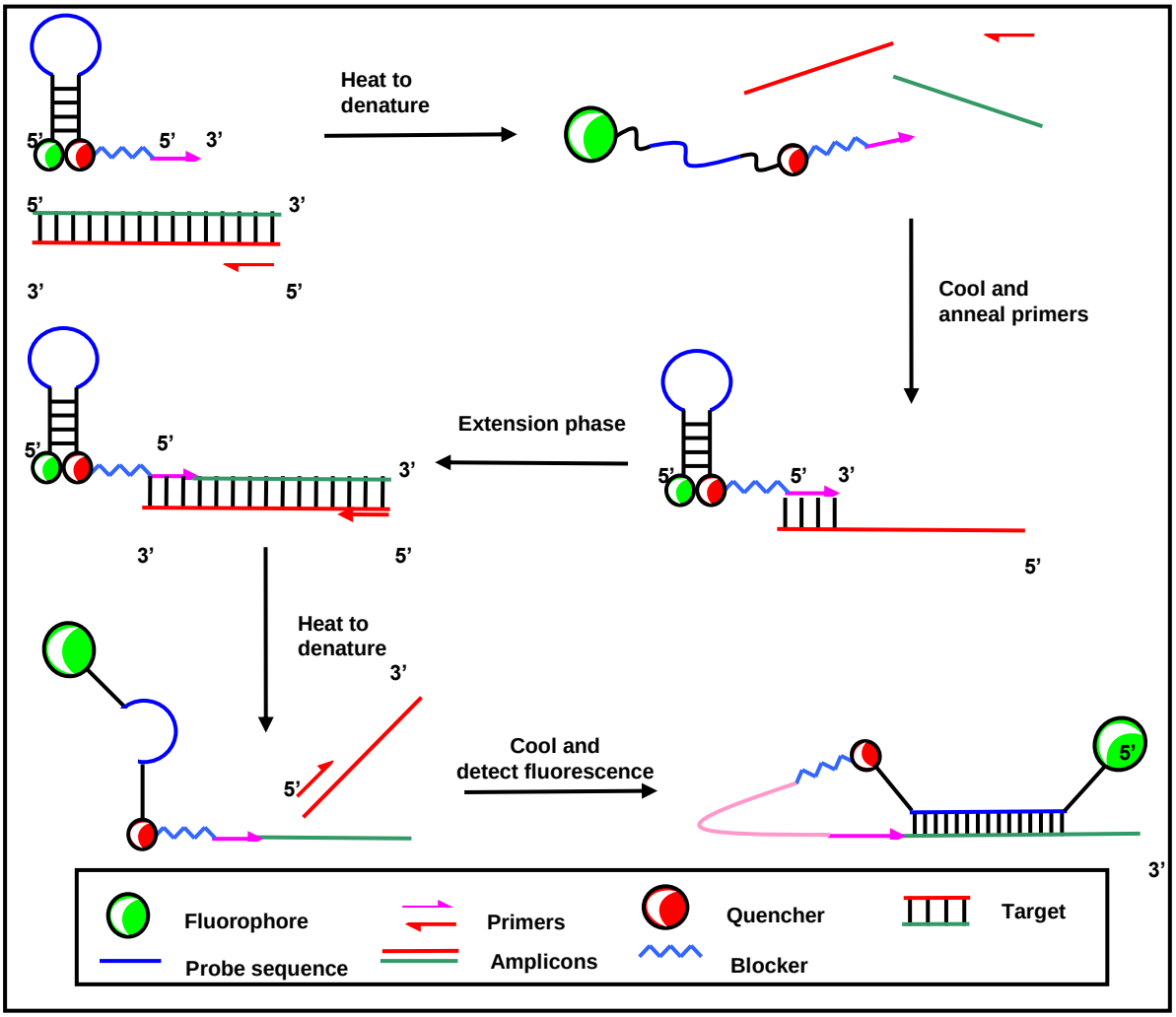


Figure A - 9 Illustration of the basis of the Scorpion® probes. During qPCR these probes are incorporated into the target sequence, providing an intramolecular detection system.

Scorpion® probes may also be used as end point detection systems because they are highly specific, and are suitable for detection of SNPs, e.g. mutant when present as a minority sequence in a background of, for example, the wild type sequence.

A.3.5.6 HyBeacons®

HyBeacons® are a simpler linear probe system which only requires a single labelled reporter. These probes have a single fluorescent label attached to an internal nucleotide, and show enhanced fluorescence on binding to a complementary target.[20]

A.3.5.7 Fluorescent labels and quencher molecules

The choice of appropriate fluorescent label and quencher for a probe depends on both the label/quencher combination and the instrument being used. The most frequently selected dyes are listed below in Table A - 2 along with the recommended quenchers. It is critical to ensure that the instrument used has the capability to excite and detect the selected dyes. All instruments are optimised for the excitation and detection of FAM and for SYBR® Green I when using the same optical conditions.
When multiplexing, dye combinations should be selected to convey excitation and emission wavelengths that are as distinct as possible from each other to minimise optical cross talk. The most usual selection is: FAM, HEX, Texas Red, Cy5. Under identical conditions it is usual to observe differences in emission intensity from different dyes. For this reason it is advisable to analyse the data from each dye combination independently (with a different threshold setting as appropriate for the probe emission).

Dye	Excitation (nm)	Emission (nm)	Quencher
SYBR® Green I			N/A
BEBO			N/A
6-FAM	494	515	TAMRA, BHQ1
Alexa 488	495	519	TAMRA, BHQ1
JOE	520	548	BHQ1
TET	521	536	BHQ1
Cal Fluor Gold® 540 (TET/JOE alternative)	522	541	BHQ1
HEX (VIC alternative)	535	555	BHQ1
Cal Fluor® Orange 560 (VIC alternative)	540	561	BHQ1
TAMRA	555	576	BHQ2
ROX	573	602	BHQ2
Texas Red	583	603	BHQ2
Cal Fluor® Red 590 (TAMRA alternative)	540	561	N/A
LC Red 640	630	640	N/A
CY5	651	674	BHQ2
Quasar 670 (CY6 alternative)	649	670	BHQ2
LC Red 700	690	710	N/A

Table A - 2 Table of frequently used dyes with appropriate quenchers.

A.3.6 Oligonucleotide synthesis and handling

When ordering custom oligonucleotides for use in PCR or qPCR, decisions must be made regarding the desired yield/scale of synthesis, purity and required modifications. Each of these factors impacts on the other; for example, a higher level of purification will result in better quality of oligonucleotide but impair the overall yield. Table A - 3 and Table A - 4 below give a general guide to the synthesis scale and expected yield. The headings in these tables refer to different oligonucleotide purification methods, which are described in section A.3.6.2.

Standard Oligonucleotides (OD/µg)*				
Scale (µmol)	**Desalt**	**Cartridge(RP1)**	**HPLC**	**PAGE**
0.03	3/90	NA	NA	NA
0.05	5/150	1.0/30	1.0/30	0.5/15
0.2	12/360	3.0/90	2.5/75	1.0/30
1.0	40/1,200	12/360	13/390	5/150
10	400/12,000	NA	130/3,900	NA
15	600/18,000	NA	190/5,700	NA

Guarantee is for 20-mers or longer. Shorter oligonucleotides may have fewer ODs.

Table A - 3 Expected yield results for standard oligonucleotides depending on purification method.

Modified Oligonucleotides (OD/µg)*				
Scale (µmol)	**Desalt**	**Cartridge(RP1)**	**HPLC**	**PAGE**
0.05	2/60	0.4/12	0.4/12	0.2/6
0.2	5/150	1/30	1/30	0.4/12
1.0	16/480	5/150	5/150	2/60

** Guarantee is for 20-mers or longer. Shorter oligonucleotides may have fewer ODs. Note: Post-synthesis modifications may yield 50% less than the above stated values.*

Table A - 4 Expected yield results for modified oligonucleotides depending on purification method.

A.3.6.1 Oligonucleotide preparation

All DNA oligonucleotides that are provided lyophilised are ready for use upon resuspension. It is recommended that oligonucleotides are resuspended in a weak buffer such as TE buffer (10 mM Tris, pH 7.5-8.0, 1 mM EDTA). In applications where TE is not suitable, sterile nuclease-free water may be used. However, high-grade water may be slightly acidic and is not recommended for long-term storage.

A 100 µM stock solution may be obtained by using the following guideline: take the number of nanomoles (nmol) provided (information found on the tube label and/or quality assurance document supplied with the oligonucleotides) and multiply by a factor of 10. The resultant number is the volume (in microlitres) of liquid to be added to the tube to achieve a final concentration of 100 µM. Note that this is equivalent to a stock solution of 100 pmol/µL. The stock solution may then be further diluted as necessary based upon the application requirements. The stock solution is stored in aliquots at -20 °C to avoid multiple freeze-thaw cycles.

A.3.6.2 Oligonucleotide purification

When DNA is synthesised, each nucleotide is coupled sequentially to the growing chain. In each coupling cycle, a small percentage of the oligonucleotide chains will not be extended, resulting in a mixture of full-length product and truncated sequences.

After the oligonucleotide is cleaved from the support and the protecting groups are removed, purification is used to separate the full-length product from the truncated sequences. In general, the purity required for a specific application depends on the potential problems from the presence

of truncated oligomers. For some applications, it is crucial that only the full-length (n) oligonucleotide be present. For others, such as PCR primers, the presence of shorter oligomers (n-1,n-2,...) may not affect the experimental results.

A.3.6.2.1 Desalting

The desalting procedure removes residual by-products remaining from the synthesis, cleavage and de-protection procedures.

For many applications, including PCR, desalting is acceptable for oligonucleotides less than 35 bases in length, as the overwhelming abundance of full-length oligonucleotide outweighs any contributions from shorter products. Oligonucleotides comprising of more than 35 bases require an additional method of purification such as Reverse-Phase Cartridge Purification (RP1) or PAGE (depending on length).

A.3.6.2.2 Reversed-phase cartridge purification (RP1)

Separation on a reversed-phase cartridge also removes a high proportion of truncated sequences. The difference in hydrophobicity between full-length product (which contains a 5'-DMT group) and truncated sequences (without DMT groups) forms the basis of the separation. While the full-length DMT oligonucleotide is retained on the column, the truncated sequences are washed off. The desired full-length product is then recovered after cleaving the DMT on the cartridge.

A.3.6.2.3 Reversed-phase HPLC

As the oligonucleotide length increases, the proportion of uncapped products (truncated sequences bearing the DMT) tends to increase. Not all of these impurities will be removed by RP1 and thus for longer oligonucleotides, such as artificial amplicon template oligonucleotides or labelled probe oligonucleotides, HPLC or PAGE purification is recommended.

Reverse-phase high performance liquid chromatography (RP-HPLC) operates on the same principle as a reverse-phase cartridge. However, the higher resolution allows for higher purity levels. HPLC is an efficient purification method for oligonucleotides with fluorophores, such as qPCR probes, as their intrinsic lipophilicity provides excellent separation of product from contaminants. Furthermore, RP-HPLC is a method of choice for larger scales due to the capacity and resolving properties of the column. The resolution based on lipophilicity will decrease as the length of the oligonucleotide increases. Therefore, RP-HPLC is usually not recommended for purifying products longer than 50 bases. Although longer oligonucleotides (up to 80 bases) can be purified using this method, the purity and yields may be adversely affected.

A.3.6.2.4 Anion-exchange HPLC

Anion-exchange separation is based on the number of phosphate groups in the molecule. The anion-exchange purification method involves the use of a salt-gradient elution on a quaternary ammonium stationary phase column or a similar structure. The resolution is excellent for the purification of smaller quantities. This technique can be coupled with purification by RP-HPLC, adding a second dimension to the separation process. Anion-exchange HPLC is limited by length (usually up to 40-mers). The longer the oligonucleotides, the lower the resolution on the anion-exchange HPLC column and therefore the lower the purity of the target oligo.

A.3.6.2.5 PAGE

The fundamental basis of the PAGE (polyacrylamide gel electrophoresis) separation relies on charge difference rather than molecular weight, leading to good size resolution, resulting in purity levels of 95-99% full-length product. Yields from PAGE are lower than from other methods due to the complex procedure required for extracting oligonucleotides from the gel and the removal of the vast majority of truncated products. This technique is recommended when a highly purified product is required. PAGE is the recommended purification for longer oligonucleotides (≥50 bases).

A.4 Assay optimisation and validation

Assay optimisation is central to ensuring that qPCR will perform well. There are a number of factors that can be altered to obtain optimum assay performance, which will lead to higher molecular sensitivity, specificity and precision. A key point is that, while assays can be purchased from a number of skilled commercial providers, they should be validated by each laboratory before they are used for analytical purposes. Suppliers' claims relating to the performance of assays should be verified in-house and it is worth noting that further optimisation may be necessary to obtain optimum assay performance.

An assay that has been designed such that all criteria were met (see Assay design, Section A.3) is likely to perform well under a wide range of conditions (see Figure A - 10 – example of primer T_m (melting temperature) optimisation). However, all assays have specified optimal conditions and these are dependent on the instrument and selected reagents (e.g..buffer conditions) as well as factors that can be further manipulated. Assay optimisation is generally restricted to primer optimisation using primer concentration and/or T_m, although occasionally magnesium concentration and ramp rate (which is change in temperature per second) are also considered.

A.4.1 Primer T_m optimisation

The kinetics of primer hybridisation can be influenced by primer concentration and T_m. When optimising assay conditions using primer concentration, a fixed T_m is selected and the optimal conditions for each primer are addressed independently. This is critical when designing an assay to be performed in multiplex and is a strategy used to rescue poorly performing assays when an alternative design is not possible. A technically simpler approach is to select a fixed primer concentration and then optimise the T_m that gives the best result for the selected combination of primers. This is the preferred approach when using several assays and a DNA binding dye detection, such as SYBR® Green I. However, this approach requires an instrument that can simultaneously run reaction programmes utilising different T_m.

A.4.2 Primer concentration optimisation

Optimisation of primer (and probe) concentration is an important step in the development of a robust assay. The lack of reproducibility between replicates and inefficient assays are indications of poor optimisation. The assay performance is usually tested with distinct primer concentrations normally within a range encompassing 50-900 nM. The combination of concentrations yielding the lowest C_q, highest technical reproducibility and a negative NTC is chosen (see Figure A - 10).

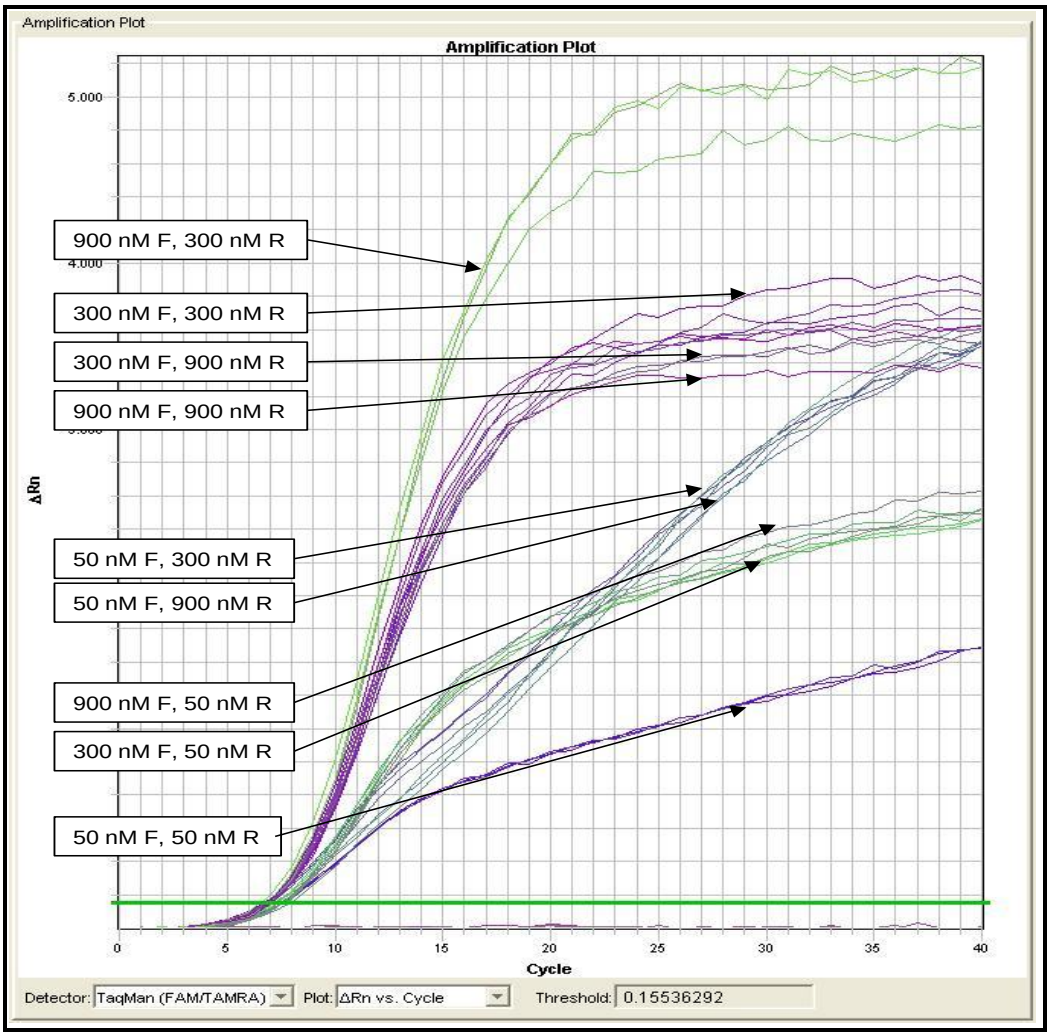


Figure A - 10 Results from a typical experiment optimising the concentration of PCR primers for a hydrolysis probe assay. Established guidelines [15] recommend that a variety of forward (F) and reverse (R) primer concentrations, usually from 50-900 nM as shown here, are tested in combination to determine the optimal concentration for the assay. In this experiment, the 900 nM forward and 300 nM reverse combination was chosen as the optimal because this combination represented the lowest primer concentrations that reproducibly yielded the earliest C_q values whilst retaining a sigmoidal curve, with very little variation between replicates (figure courtesy of Sigma-Aldrich®).

A.4.3 Cycling conditions and primer annealing temperature

qPCR assays are generally performed using two- or three-step temperature cycling programmes, typically with 35 to 40 cycles. Two-step reactions cycle between two temperatures, most usually 95 ^{o}C (typically for 10-15 seconds) and 60 ^{o}C (typically for 30-60 seconds, or 5-10 seconds under fast conditions). Two-step temperature reactions are ideal for performing many different assays using the same parameters, which may be a consideration when conducting high throughput diagnostic analyses. The disadvantage of using the two-step method is that it reduces scope for primer design and limits assay optimisation to primer concentration alone, as no T_m optimisation is possible.

A three-step reaction may be preferable when the target sequences are complex, and either the chosen primers are difficult to optimise, or if the detection system does not utilise a hydrolysis probe. Three-step strategies cycle between: 95 ^{o}C (typically for 10 seconds), an annealing temperature (between 55 ^{o}C and 65 ^{o}C, typically for 10-20 seconds or 5 seconds under fast conditions) and 72 ^{o}C (typically for 20-30 seconds or 15-20 seconds under fast conditions). In this case the T_m may be optimised to further improve assay performance using the following protocol:

- Start at the low end of the melting temperature (T_m) range to be tested, which is determined by the T_m of the primer, and increase the temperature stepwise in gradual increments (usually testing between 55 ^oC and 65 ^oC). Instruments will often have gradient blocks that facilitate temperature optimisation in a single experimental run.

- Test each reaction product for specificity either by post-PCR melt curve analysis or agarose gel electrophoresis.

- The optimal annealing temperature is the one that results in the lowest C_q with a negative NTC and a specific melt curve analysis.

If the annealing temperature is too low, the reaction will be non-specific. However, if the temperature is too high, the stringency may affect reaction efficiency, resulting in a lack of amplification, very poor yields and low reproducibility.

Although most commercial kits are supplied with standard PCR assay conditions they may, at times, require further optimisation. This would usually be due to persistence of primer-dimers, non-specific amplification or suboptimal reaction efficiency. Upon completion of optimisation, assay efficiency should be calculated by measuring a series of standards and preparing a standard curve (see Standard curve, section A.4.5.1.1). It is possible that, even after optimisation, the efficiency may still be suboptimal.

The criterion for “acceptable” efficiency should be defined by the user prior to beginning the optimisation process. Different users have distinct requirements. Ideally, the efficiency should be >90%. However, it is possible to perform accurate measurements with assays exhibiting <90% efficiency. As with primer design, the target sequence may dictate that only a reduced efficiency is possible. Nevertheless, when using an assay with a lower efficiency it is likely that precision and the limit of detection will be affected. While this does not preclude the use of assays with lower efficiencies, it is important that users are aware of any additional limitations through assay validation (see Assay Validation, Section A.4.5) and use appropriate discretion when interpreting data.

A.4.4 Magnesium chloride

The concentration of $MgCl_2$ has an impact on both the specificity and yield of qPCR as magnesium affects the hybridisation of the primer to the target, the processivity of *Taq* polymerase, as well as the rate of hydrolysis by the exonuclease moiety. Hence, insufficient $MgCl_2$ results in poor yields as the polymerisation rate of *Taq* polymerase is low, primer binding is compromised, and probes are not cleaved efficiently. If the level of $MgCl_2$ is too high the specificity of the reaction is compromised.

In contrast to conventional PCR assays which use 1.5-2 mM standard $MgCl_2$ concentrations, hydrolysis probe assays require higher $MgCl_2$ concentrations of around 3-5 mM to achieve efficient cleavage of the probe. The presence of $MgCl_2$ also increases the rate of DNA hybridisation, enabling efficient hybridisation during the rapid cycling conditions employed in many instruments.

A.4.5 Assay evaluation

Once the assay is optimised, with the identification of the most sensitive and specific conditions, it is important to determine the assay efficiency and technical dynamic range.

The most effective means to measure assay performance is via the construction of a standard curve from a serial dilution of template. Assay efficiency can be measured as a factor of the standard curve gradient. The technical assay dynamic range is determined by running a wide range of sample concentrations, ensuring that these reach a limiting dilution. In this way the limit of detection may be established.

Alternative approaches that evaluate assay efficiency within a single reaction have been proposed.[21,22] These approaches rely on algorithms to model the amplification plot curves and so are dependent on the number of cycles over which there is an increase in fluorescence. These are most likely to succeed when the measurement is made using a DNA binding dye since these assays yield a greater change in fluorescence. While this type of approach potentially offers an ideal alternative to the implementation of a standard curve, the latter is still the more common method used for assay evaluation. This is because standard curves not only provide an estimation of efficiency, they also provide additional information about working range and are conceptually easy to apply.[23]

A.4.5.1 Quantification strategies: Standard curve quantification versus comparative quantification

The quantities of target sequences within a test sample are determined in relation to the concentration of the same target in one or more control samples. These controls consist of either another sample(s) that can be regarded as a reference sample, or a dilution series of a target that is then used to construct a standard curve.

Note on absolute and relative quantification*: classically, the terms absolute and relative quantification have been applied to performing qPCR with or without a standard curve, respectively. However, as both of these methods are essentially relative quantification (relative to a standard curve or another sample), this has led to considerable confusion within the qPCR community and has been addressed in the MIQE guidelines. This qPCR guide follows the recommendations in the guidelines and therefore avoids the use of absolute and relative quantification terms.*

Standard curve quantification strategies use a standard or calibration curve as a control or reference. The quantities of target sequences in all samples are calculated in relation to a serial dilution of template material incorporated in a standard curve. For standard curve quantification, the curve is constructed using a template containing a defined copy number of template molecules, most usually an artificial oligonucleotide or linear plasmid containing a clone of the target. Standard curve quantification can also utilise template material of unknown concentration, such as a preparation of cDNA or gDNA.

A.4.5.1.1 Standard curve

A standard curve is commonly generated using a ten-fold serial dilution series of at least five template concentrations. It is best practice to run each reaction in triplicate, particularly during optimisation steps. It is critical that the quantities of target in the experimental samples should fall within the concentration range covered by the standard curve.

After detecting the target in each standard sample, the standard curve is plotted as C_q vs. logarithm of template concentration (Figure A - 11). This is an automatic function in most qPCR instrument software packages.

The assay efficiency is calculated from the slope (m) of the best fit straight line through the standard curve data points, described by the equation (2):

$$y = mx + c \quad (2)$$

where efficiency is calculated as (3):

$$E = 10^{(-1/m)} - 1 \quad (3)$$

The efficiency of an assay should be a value close to 1, with 1 indicating a 100% efficient reaction.

The correlation coefficient (r^2) provides an estimate of the goodness of fit of the data points to the linear trendline. For a well-optimised reaction all three technical replicates should be highly

reproducible and r^2 should be close to 1 (and greater than 0.98), when a dilution series ranging over 7 logarithms is measured.

The intercept (c) of the standard curve on the *y*-axis gives a theoretical sensitivity of the assay, indicating the number of cycles required to detect a single unit of measurement (from *x*-axis).

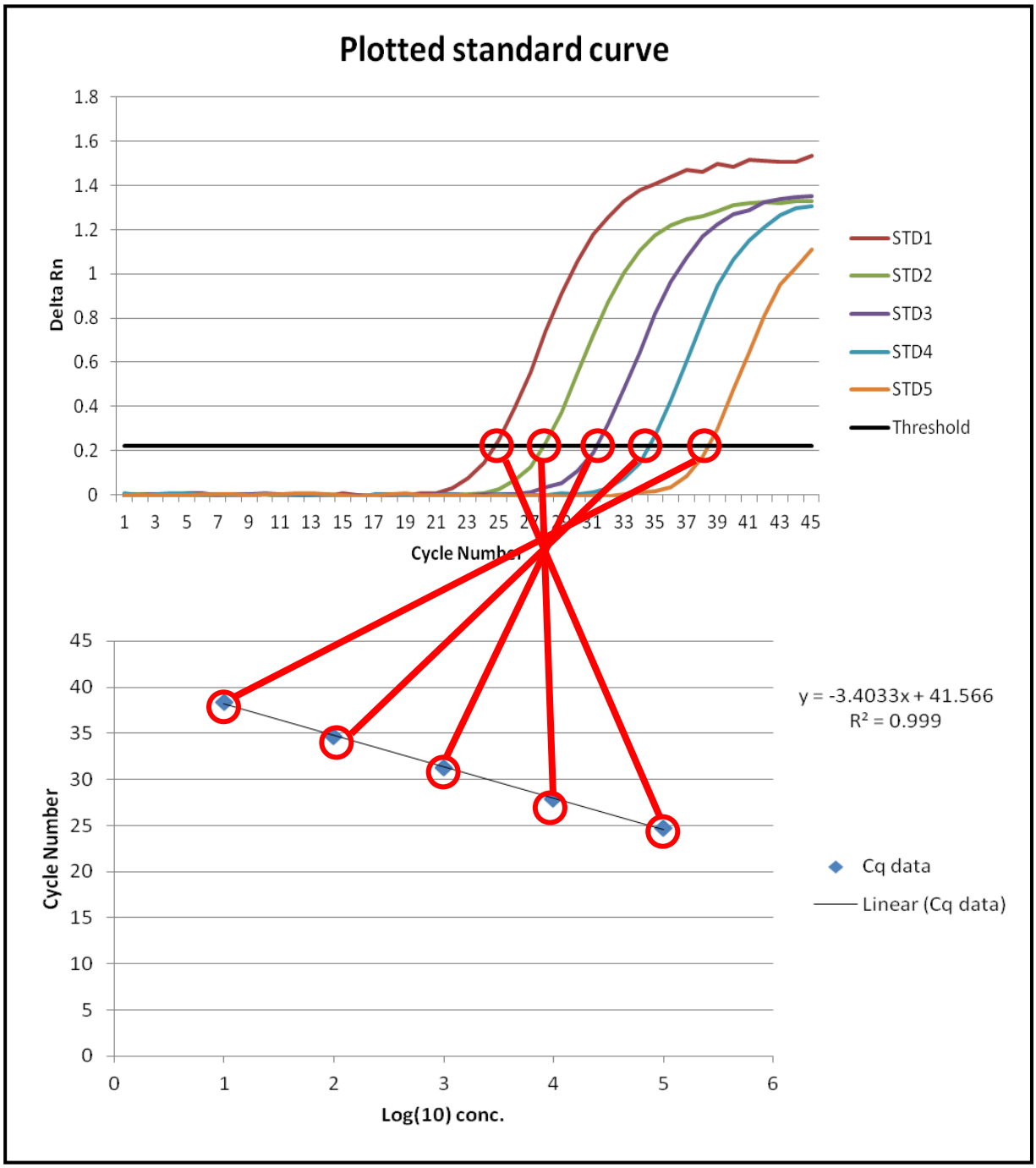


Figure A - 11 Standard curve - five serial dilutions. The Cq values for the five standard solutions are plotted against the log of the concentration to produce the standard curve.

The standard curve is a plot of measured C_q against the log of the target concentration. As the measurement of each concentration point is performed in triplicate, these data provide information on the precision of the assay. Assay efficiency and repeatability can be predicted from the line of best fit: amplicon accumulation is proportional to 2^n, where n is the number of amplification cycle repeats. Therefore:

- 2^n = fold dilution,
- 2 fold dilution n~1,
- 10 fold dilution n~3.323,

Therefore when a 10 fold serial dilution is performed, the amplification plots for each dilution should be 3.3 cycles apart.

Standard curve quantification method

In this method, the relative quantity of a gene of interest (GOI) is measured and normalised (see Assay normalisation, section A.5.2) to that of a validated reference gene (RG) (see Reference gene selection, section A.5.2.1).

To apply this method (see completed example in Table A - 5):

1. Generate standard curves using the calibrator for the GOI and the RG. These may be performed individually or be a multiplex assay using a single serial dilution of cDNA or gDNA;
2. Calculate the target concentration for the reference gene for each unknown sample by comparison to the RG standard curve;
3. Calculate the target concentration for the GOI for each unknown sample by comparison to the GOI curve;
4. Normalise GOI to RG (GOI/RG).

When using this analysis option, standard curves are required for every experimental plate.

Sample	GOI Copy no [1]	Reference gene (RG) Copy no [2]	Normalised GOI/RG=[1]/[2]= [3]	Relative Values [3]/[A3]
A	1198	34089	0.0351	1.000
B	1836	8326	0.2205	6.280
C	2959	2357	1.2554	35.76
D	11	955	0.0115	0.328
E	39	6447	0.0061	0.170

Table A - 5 Example of a standard curve quantification method.

A.4.5.1.2 Comparative quantification

Comparative quantification is used to measure the relative change in expression levels between samples under different experimental conditions or over a period of time. The concentration of the GOI in each sample is compared to a validated reference gene or multiple reference genes, to normalise for variations in sample loading (see Reference gene selection, section A.5.2.1).

The reference gene(s) may be co-amplified in the same tube in a multiplex assay, or amplified within separate tubes. The quantification value is expressed as a ratio between the GOI concentration in all unknown samples and GOI in the calibrator. Therefore, the results are expressed as ratios relative to the control sample or calibrator, which is defined as 1.

Delta delta Cq (2-ΔΔCq) or comparative quantification method

The delta delta C_q (termed as $2^{-\Delta\Delta Cq}$), also known as the comparative quantification method, relies on a direct comparison of C_q values and was proposed by Livak and Schmittgen.[24] When using the delta delta C_q, a standard curve is ideally prepared during the optimisation steps of the experiment in order to verify the reaction efficiencies.

It is essential that the amplification efficiencies of the GOI and the RG(s) are virtually identical, and close to 100%, when using $2^{-\mathbf{\Delta\Delta Cq}}$ analysis. For subsequent analyses of samples it is then assumed that the assay efficiency is constant (as determined during optimisation), and the C_q of the GOI is subtracted from the C_q of the selected RG(s). As the RG is assumed to be constant (albeit the assumption must be validated; see Normalisation, section A.5), this normalises against differences in the GOI C_q that are attributable to technical error, ideally leaving only biological variation to be measured.

This approach, while popular, does have two potential problems. Firstly, the incorrect assumption of equal PCR efficiencies may lead to data inaccuracy. Secondly, the strategy of comparing the C_qs from different assays is problematic as the C_q is an arbitrary value rather than a defined unit. The C_q value can only be directly compared between different assays if the amplification plots exhibit the same shape, and ideally, if all samples are loaded onto the same plate.

A preferable method would take the efficiency into account in the calculation and compare the C_q difference of the reference gene(s) between the sample of interest and the calibrator (control) sample, with the C_q difference of the GOI between the sample and the calibrator. The equation as described by Pfaffl *et al*. [25] is shown below (4):

$$\text{ratio} = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}} \qquad (4)$$

A.4.6 Assay and method validation

Up to this point we have referred exclusively to the design, optimisation and evaluation of assays. However, for any method (which may encompass these assays) to produce meaningful and reliable data, some performance checks should be made prior to the analysis of real samples. The validation process typically entails understanding the reason the measurements are being made, and producing data to demonstrate that the method performance is fit-for-purpose. For qPCR it is important to note that assay performance will differ between different primers sets and can even differ between different syntheses of the same primer pair. Ideally, the validation study will need to cover all the steps in the method, from sampling and sample storage through to data analysis, to ensure evaluation of all parameters that may influence the result.

The actual level of assessment and validation that is undertaken will depend on the intended use of the method and the importance of the data produced. For qPCR such comprehensive validation is not commonly performed at present, with the exception of applications such as clinical diagnostics or genetic analysis for food authentication purposes. However, at the very least, users should consider validation of the qPCR step (and the reverse transcription when performing RNA analysis). There are many performance parameters that may potentially be investigated for a particular method; hence, choosing the characteristics to be investigated is an essential part of the validation process. For quantitative work, evaluating random error (precision) and systematic error (method bias) is crucial to achieve accurate reproducible measurements. Unless working at trace levels, establishing the limit of detection (LoD) and quantification (LoQ) may not be needed, although it is important to know the range over which the method response is linear. A well optimised qPCR can be precise over a large dynamic range, but it is important to confirm this empirically.

Performance parameters are briefly listed in the following section; their inclusion in the validation study will need to be evaluated in the method validation planning stage. Further information about the type of experiments that can be performed to evaluate these parameters can be found in "Essentials of Nucleic Acid Analysis" RSC Publishing, Edited by Jacquie T Keer and Lyndsey Birch (2008).[26]

A.4.6.1 Precision

Precision is defined as the closeness of agreement between independent test/measurement results obtained under stipulated conditions. Measurements for precision estimates should be performed on identical samples, and expressed as the standard deviation or relative standard deviation of the results obtained from the precision study.

The conditions under which the repeated measurements are made will determine the type of precision estimate obtained. Three common types are repeatability, reproducibility and intermediate precision. It should also be noted that all the steps required to make a qPCR measurement will contribute to the random error and may also contribute to the systematic error. Consequently for the most accurate measurements the whole process from sampling to qPCR needs to be considered.

A.4.6.2 Bias

Bias is defined as the difference between the expectation of a test result or measurement result and an accepted reference value. Bias is a measure of the trueness of a result and is caused by systematic errors, rather than the random errors which influence the precision of results.

Bias is calculated as the difference between the observed averaged value from the study and the accepted reference value of the test sample, and is often expressed as a percentage difference from the expected reference value.

A.4.6.3 Ruggedness

Over time experimental parameters will vary to some extent, with a possible effect on the method performance. Ruggedness or robustness testing helps identify those parameters which have a significant effect on the performance of a method and provides useful information on how closely such parameters need to be controlled. A rugged or robust method is one that exhibits a performance unaffected by changes in the experimental parameters within the defined control limits.

To evaluate ruggedness, experiments which deliberately and systematically introduce known changes to parameters are designed, and the effect assessed. This approach can be streamlined and simplified using experimental design tools such as "Placket and Burman" design.[27]

Parameters identified as having a significant effect require further study to set suitable control limits.

A.4.6.4 Selectivity (Specificity)

Selectivity is the extent to which the method can be used to determine the target analyte without interferences from other components of similar behaviour. The selectivity of the assay is determined at the "Assay design and optimisation" stage. However, it is recommended that the whole method is assessed for its selectivity by performing measurements on samples which contain the target analyte and known impurities to ensure performance is not compromised at any stage.

A.4.6.5 Detection limit (Sensitivity)

It must be noted that while in biological analysis 'sensitivity' is used to describe the lowest concentration of target analyte that can be measured, it has a different definition in other fields, so it is important to follow specific sector guidelines where available.

Sensitivity is often determined as part of the method validation and gives an indication of the lower operating limits of the method. Most experiments to determine detection limits require a replicated sequence of experiments on low-concentration samples, blanks (negative controls) or low-concentration spiked materials or standards.

A.4.6.6 Working range and linearity

The working range is the interval between the upper and the lower concentrations of an analyte that has been determined suitable for the method. The term linearity is frequently linked with the working range of the method and refers to the ability of a method to give a response that is directly proportional to the concentration of the analyte.

In order to establish the working range of a method it is necessary to study the response of standards (or where standards are not available, in-house samples or spiked samples) whose concentrations span the range of interest.

Evaluation of linearity can be performed visually and is supported with objective regression data.

A.4.6.7 Measurement uncertainty

Measurement uncertainty can be defined as an estimated range of values within which the true value of the measurement resides. The range of values gives an indication of the reliability of a measurement result.

Experiments performed in method validation often provide information which can be used in evaluating measurement uncertainty. In the assessment, all possible sources of variability in the measurement process are considered and evaluated to create an uncertainty budget. The uncertainty estimate will therefore include the effect of both random and systematic errors.

The experimental result can then be reported as $x \pm y$, where x is the reported measured value and $\pm y$ is the degree of uncertainty associated with the measurement result.

A.5 Normalisation

There are two factors influencing the accuracy of the results obtained from a qPCR assay. The first is the biological variation, which comprises any true change in RNA expression, as well as the normal variation in the expression of that RNA. Since the majority of experimental work aims to evaluate this biological variation, it is important that the second factor, technical variation, is minimised.

Normalisation is the process by which the impact of technical variation, or noise, is removed or reduced to facilitate data analysis of true biological variation. There are many methods for normalising technical differences, although some are better than others. It is worth noting that the application of some normalisation techniques may be more detrimental to the overall analytical process than not applying any normalisation approach.

A.5.1 Normalisation approaches

Ideally, normalisation methods need to take into account variability from the entire multi-step process that constitutes qPCR analysis (see Figure A - 12). However, applying normalisation at any one stage in the process may not control for technical error and/or bias at an earlier or later stage. Normalisation methods tend not to be mutually exclusive; hence a combination is recommended.[28] At the earliest stages of experimental design, ensuring similar sample sizes may seem an obvious consideration, yet this crucial consideration might not be as simple as it first appears. Biological tissues can be highly heterogeneous, especially when healthy tissue is compared with the diseased form, and even apparently less complex tissues such as blood can differ considerably in their cell count and composition.

Cell culture experiments are relatively easy to normalise on the basis of cell count. However, when compared with a control culture, the treatment evaluated may impact on cell morphology, complicating cell number estimations, or may result in the production of extracellular matrix giving rise to different extraction efficiencies.

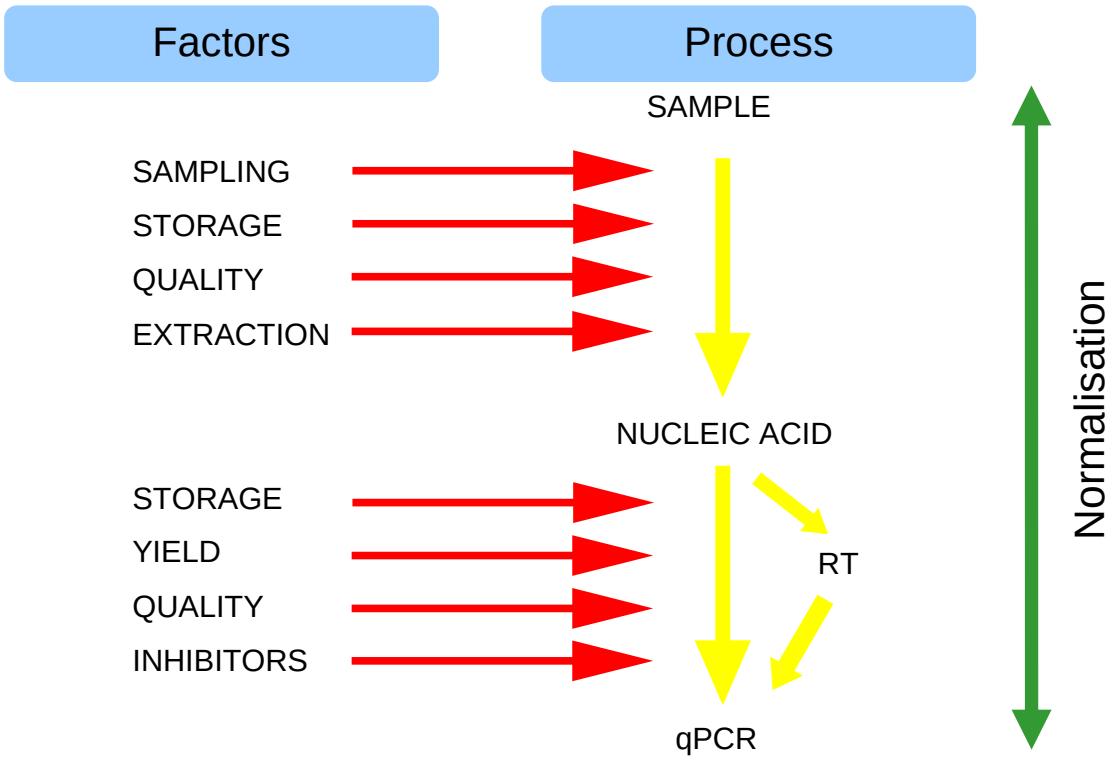


Figure A - 12 Factors that can affect a qPCR assay.

The extraction step (discussed in section A.2, Sample purification and quality control) is a major source of technical variation within the whole process, and it is important that some assessment of the resultant extract is performed to allow for accurate quantification. As a minimum requirement, an estimation of template concentration (DNA for qPCR or RNA for RT-qPCR) is important so that similar quantities of nucleic acids are compared between distinct samples. Unfortunately, as with many other procedures, this estimation may not be sufficient to remove all potential biases.

There are two types of procedures used routinely for estimation of nucleic acids: absorbance based and fluorescence based methods. Absorbance measures the amount of light that is absorbed at A_{260}, while fluorescence uses dyes, which bind to the nucleic acid, that emit light at a given wavelength (Section A.2.2, Extract quality control). Absorbance is a simple method for which there are numerous instruments available, although it is notoriously inaccurate, and affected by the presence of impurities.[3] Fluorescence is arguably more accurate when comparing two different samples and is more sensitive than absorbance. However, unlike absorbance, fluorescence measurements require a calibration curve to assign a value to a signal. This calibrator, in turn, requires a value assignment that is usually conducted using absorbance measurements.

As well as estimating nucleic acid quantity, some estimation of quality is also recommended, although this in itself presents a challenge. Generally, nucleic acid quality is evaluated by electrophoresis using agarose or acrylamide, and degradation is assessed by determining the apparent size of the nucleic acid within the resultant gel bands. The extent of implementation of this analysis is dependent upon the nucleic acid in question; it is frequently used for plasmid extracts and total RNA.

A.5.2 Assay normalisation

Ensuring that a similar amount/quality of nucleic acid is added to a qPCR or reverse transcription reaction is an important consideration, but is usually not sufficient on its own for ensuring reliable measurements. In addition, some form of amplification control is necessary.

Internal or external amplification controls may be used for assay normalisation. External amplification controls (or spikes) are used for evaluating the majority of the technical error. They are useful as the quantity of template is known and hence bias can be measured. External spikes can also be used as process controls to evaluate specific steps, such as the effect of a sample on the qPCR reaction (as described for example in the SPUD reaction, sectionA.2.3.2). Unfortunately, external spikes cannot be used to assess the efficiency of the initial lysis stage during the extraction process. Therefore, an internal amplification control (such as Alu, for example) is required to assess the impact of this crucial reaction step on the nucleic acid quantification.

When measuring DNA copy number variation for a sequence of interest known to exhibit variability, measurement is simply normalised by targeting a region of the sequence that is not subject to change. An example of how this application is the measurement of Human Epidermal growth factor Receptor 2 (HER-2) genomic amplification.[29] HER-2 genomic instability is an important prognostic indicator in breast cancer and accurate measurement of HER-2 amplification status is important in patient management. HER-2 status can be measured by qPCR by comparing the copies of HER-2 with another genomic target that is acting as a control.

For RNA normalisation, reference genes are routinely used. However, their correct selection and application has represented a major problem, as discussed in the next section.

A.5.2.1 Reference gene selection

Reference genes are essentially RNA transcripts that do not change during the course of the experiment performed. So, for example, if the purpose of the experiment was to assess the effect on the expression of gene X by the addition of a mitogen to a cell monolayer, then the expression of another gene (or genes) known not to be affected by the mitogen in question is also measured.

This provides the researcher with the immediate challenge of identifying an RNA that is not affected by the experiment (treatment) before conducting the study. This process of validation of reference genes, which may be perceived as cumbersome, is fundamental for an accurate measurement of the GOI.

RNA quantification by RT-qPCR has been routinely compromised by the incorrect choice of reference genes. The relatively common practice of taking a reference gene that is used by another laboratory in a different experiment is not acceptable. Reference genes need to be validated under specific experimental scenarios to be assured that the reference gene in question is not affected by the specific experiment performed. If this validation is not carried out, and the reference gene is affected by the experiment, this can lead to completely incorrect results and subsequent misinterpretations.[30]

There is a range of scientific literature describing different methods for normalisation,[31,32,33] as well as a plethora of publications describing the protocols required to identify the most appropriate normalisers for a given experimental scenario. Previously, choosing whether to select a single or multiple reference genes was a key consideration. However, the increased availability of normalisation kits with lower running costs has led to the current practice of routinely measuring multiple reference genes. This approach, when performed correctly, provides not only more robust findings but also facilitates a more precise measurement that is not achieved when using a single reference gene.

Essentially, selection of reference genes requires the analyst to evaluate a number of candidate targets. The basic approach is described by Dheda *et al.* 2004.[31] For normalisation using multiple reference genes this basic approach may be used in combination with the different methods available using programs such as Genorm[32] or Bestkeeper.[33] There are a number of commercially available kits that allow a simple screen of a number of targets to select appropriate reference genes prior to use in the final experiment.

A.5.3 Alternative methods

While reference genes are the favoured method for assay normalisation, there are alternatives. When measuring large numbers of targets the analyst can estimate the global mean and identify RNA sequences that deviate from this parameter.[34,35] This approach, commonly used for gene expression arrays, is a valuable alternative to using reference genes and arguably preferable where multiple targets are measured.

Another recently explored approach is the measurement of endogenously expressed repeat elements (ERE) that are present within the untranslated regions (UTRs) of many of the mRNAs. Many species contain these repeat elements (ALU in primates, B elements in mice), which can provide an estimation of the mRNA fraction, and have been shown to perform well as normalisers.[36]

A.6 Data analysis

A.6.1 Baseline correction

qPCR measurements are based on amplification curves that are sensitive to background fluorescence. The background fluorescence may be caused by a range of factors which include choice of plasticware, remaining unquenched probe fluorescence, signal carryover into the neighbouring sample wells, etc. In well-designed assays the background is low compared to the amplified signal. However, variation in background signal may hinder quantitative comparison of different samples. Therefore it is important to correct for baseline variations.

A common approach is to use fluorescence levels at early cycles, such as cycles 3-10, to identify a constant and linear component of the background fluorescence and thus normalise the amplification curve. Due to transient effects, it is advisable to avoid the first few cycles for normalisation. With the use of more cycles for the baseline correction, the potential accuracy of the linear component of the baseline variations is improved. However, higher cycles are anticipated to experience increased fluorescence due to target amplification and are therefore unsuitable for baseline normalisation.

A.6.2 Setting a threshold

Some researchers advocate mapping large parts of the amplification curve to estimate target quantities in measured samples.[37,38] However, the threshold method has proved to be an effective as well as simple quantification method.

The underlying principle of the threshold method is that information related to the target quantity is available in the log-linear phase of the amplification curve. By simply detecting the cycle where the log-linear phase of the amplification curve crosses an arbitrary threshold level, information about relative target quantities in different samples is obtained.

It is important that the threshold is set at a fixed level for all samples that are to be compared. With this proviso, the threshold can in principle be placed anywhere on the log-linear phase of the amplification curve.

In practice, the shape of the log-linear phase of the amplification is disturbed by the background fluorescence baseline at the lower amplification levels. The curve shape is influenced at higher amplification levels by the plateau phase where the finite amounts of PCR reagents and oligonucleotides limit amplification. It is recommended to set the threshold as follows (see example in Figure A - 13):

- Sufficiently above the background fluorescence baseline to be confident of avoiding having any amplification curve cross the threshold prematurely due to background fluorescence, and
- As low as possible to ensure that the threshold crosses at the log-linear phase of the amplification curve where it is unaffected by the plateau phase.

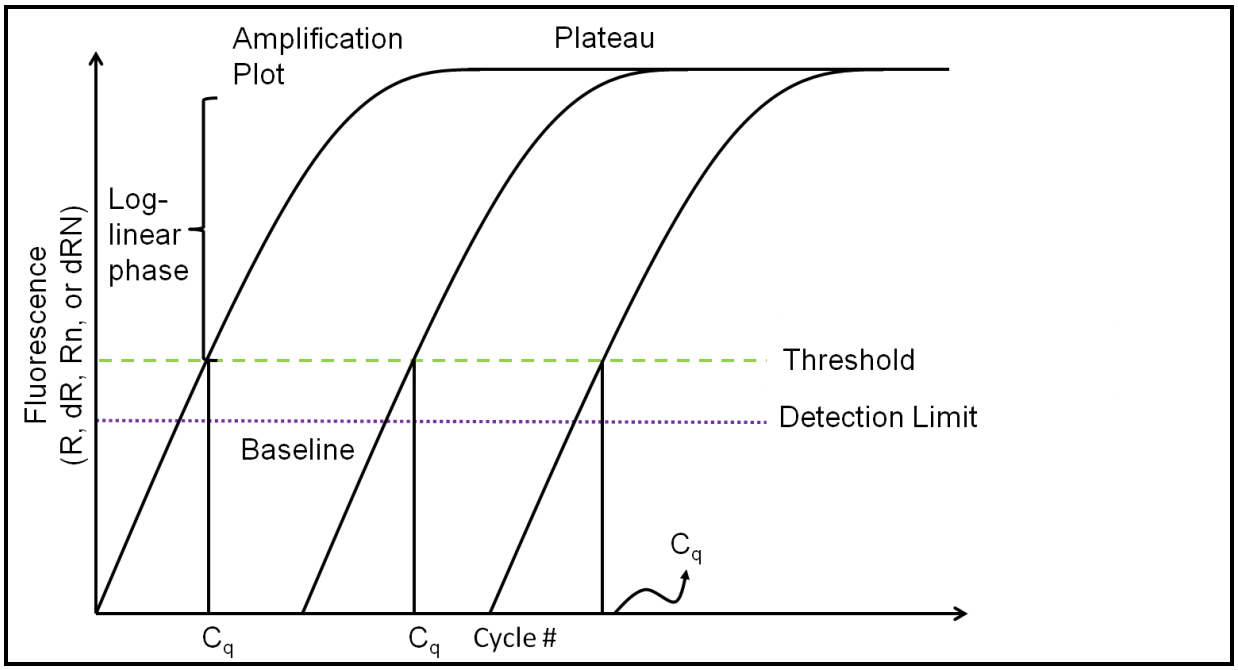


Figure A - 13 Amplification curves. This graph shows the increase of fluorescence with the number of cycles for different samples. The threshold is set above the detection limit but well below the plateau phase where the amplification rate slows down.

A.6.3 Essential biostatistics

The analysis of qPCR data is subject to interpretation. In the interest of scientific integrity it is important that the interpretation is based on a solid foundation of defined statistical significance criteria and that this is clearly documented within publications. Furthermore, in order to observe biological effects, any change caused by the effect(s) of interest needs to be of large enough magnitude to stand out above the experimental variability generated by the technical process. However the analyst must also be aware of bias. As the technical error is reduced, the chance of measuring experimental bias as an incorrect significant finding increases. This effect can be mitigated by replicating the experiment correctly. A classic example of this erroneous approach is illustrated when performing RNA analysis with replicates of the PCR step but without replication of the more variable reverse transcription reaction.

Significance testing is an important statistical tool for data evaluation. A significance test allows an objective judgement to be made regarding observed differences between sets of results. Are these observed differences due to a 'real' effect, or could they be accounted for by the random error inherent in the qPCR process? Before embarking on any experimental study, the analyst should have a clear understanding of the intended objective of the experiment. The strategy for data analysis should also be planned beforehand. Significance testing requires the formulation of hypotheses which will relate directly to the aims of the experiment. The usual starting point is to assume that there is no effect (e.g. the expression of a gene is unaffected by a particular treatment and there is therefore no significant change in observed C_q values). This is referred to as the null hypothesis, H_0. A second, alternative, hypothesis (H_1) is also required. This describes a possible alternative outcome of the experiment (e.g. the expression of a gene is influenced by a particular treatment and there is therefore a significant change in C_q values). It is important to remember that the null hypothesis is assumed to be true, until the data indicate otherwise (i.e. the burden of proof is on H_1). Using significance tests (e.g. a Student *t* test for comparing means of data sets), it is possible to calculate the probability that the observed difference (or one more extreme) arises solely by chance, *if the null hypothesis is true*. The lower the probability, the less likely it is that the null hypothesis is true. A threshold is set so that the null hypothesis will be rejected when the probability drops below a certain level. It is important to remember that there will always be a chance that the null hypothesis will be rejected when it is in fact true (a false positive or Type I error). The threshold is set so that the probability (α) of a Type I error is

controlled to an acceptable level. This is also referred to as the significance level for the test. Typically a value of α=0.05 is used. Therefore, if the probability (*P*) obtained for a particular significance test is less then 0.05, the null hypothesis is rejected and the result of the test is deemed as significant. However, for α=0.05 the chance of incorrectly rejecting the null hypothesis will be 5% (1 in 20).

The analyst also needs to be aware of the possibility of a Type II error (accepting the null hypothesis when it is, in fact, false). The probability of not committing a Type II error is called the 'power' of the test. The power of a test depends on several factors including: 1) the size of experimental effects being studied, 2) the statistical significance criteria, 3) the magnitude of variability in experimental measurements and 4) the sample size.

The size of the experimental effect is often beyond the control of analyst. A lot can be accomplished by a skilled analyst to minimise variability due to technical handling in experimental measurements. In previous chapters, many examples of techniques aimed at minimising variability have been discussed, including assay optimisation, normalisation, use of technical replicates and more. However, the factor that has arguably the greatest impact on the statistical power is the sample size, that is, the number of biological replicates used in the study.

In practice, economic and practical considerations limit, sometimes severely, the number of biological replicates that are accessible for a study. In these cases it is particularly important to design the experiment in an appropriate manner to avoid bias or even erroneous conclusions. A common pitfall with experimental design relates to bias and loss of statistical power from multiple testing issues.

A.6.3.1 Multiple testing issues

The discussion of Type I errors above is relevant for a single test. However, the situation becomes more complex when multiple testing is required. For a single test with a significance level α=0.05, there is a 5% chance of incorrectly rejecting the null hypothesis. The probability will be greater than 0.05 if multiple tests are carried out. Multiple testing therefore refers to the fact that a statistical test with several independent null hypotheses is more likely to yield a significant result than a single test, and that the chances of this increases with the number of null hypotheses tested, even if the underlying probability distributions are identical.

For example, consider an experiment where the expression of 10 genes was measured in response to a treatment. A statistical test comparing treated and control samples for a single gene, at a 0.05 significance level, will have a 5% risk of incorrectly showing a significant effect (i.e. a Type I error). Remember that while 5% or α=0.05 is the most commonly used significance level when performing biological analysis, this 1 in 20 chance of a Type I error is not terribly stringent. If *m* tests are carried out, each with a significance level α, the experiment-wide significance level α' (i.e. the probability of obtaining one or more significant results by chance) is given by equation (5):

$$\alpha = 1-(1 - \alpha)^{m} \qquad (5)$$

Therefore, if the same test (at a 0.05 significance level) is repeated for all 10 genes in a study, the overall risk that at least one of the tests yields a false positive result is $(1-(1-0.05)^{10})=0.4$ (40%) (see Table A - 6) So, in order to maintain the overall risk of a false positive result at 5%, a compensation for multiple testing is required. This is achieved by making the significance criteria for each individual test more stringent, i.e. by reducing the significance level α. A commonly applied approach is the Bonferroni correction shown in Table A - 6. In the case of studying 10 genes, the number of independent null hypotheses (*m*) is 10. Therefore, according to the Bonferroni correction, to maintain the overall risk of a false positive result at 5% requires a significance level for each test of only α=0.0051 (i.e. 0.51%). At the corrected significance criteria, a larger sample size may be required in order to maintain statistical power. It should be noted that

in some situations, the Bonferroni correction is very conservative and other approaches may be more suitable.[39]

However, arguably a bigger concern is that the analyst may perform multiple testing of alternative hypotheses without realising and without making proper corrections. For this purpose, adhering to a defined operating procedure may be helpful.

Number of Independent Null Hypotheses (*m*)	Probability (P*) of obtaining one or more P values less than 0.05 by chance	Significance level (α) for individual tests required to keep overall Type I error rate equal to 0.05
1	5%	0.0500
2	10%	0.0253
3	14%	0.0170
4	19%	0.0127
5	23%	0.0102
6	26%	0.0085
7	30%	0.0073
8	34%	0.0064
10	40%	0.0051
20	64%	0.0026
50	92%	0.0010
100	99%	0.0005

Table A - 6 Bonferroni correction for multiple statistical comparisons

A.6.3.2 Exploratory and confirmatory approaches to statistical analysis

In the context of statistical data analysis, a distinction is made between exploratory and confirmatory studies (see Figure A - 14). The purpose of the exploratory study is to analyse data with one or several different techniques in order to generate one or several plausible hypotheses.

Since in an exploratory study no attempt is made to test a hypothesis, the rigor of the statistics used is of secondary importance. The primary focus is to disclose structures in the data that may form plausible hypotheses and for this purpose, data may freely be re-analysed in an iterative process to explore the data for plausible hypotheses.

In contrast, the purpose of the confirmatory study is to determine whether a specifically formed, prior hypothesis should be rejected or accepted based on evidence accompanying the experimental data, employing predetermined criteria of inferential statistics. The confirmatory approach thus requires a well formed and strictly limited hypothesis *before* data collection. By strictly adhering to the confirmatory approach, multiple testing issues may be avoided.

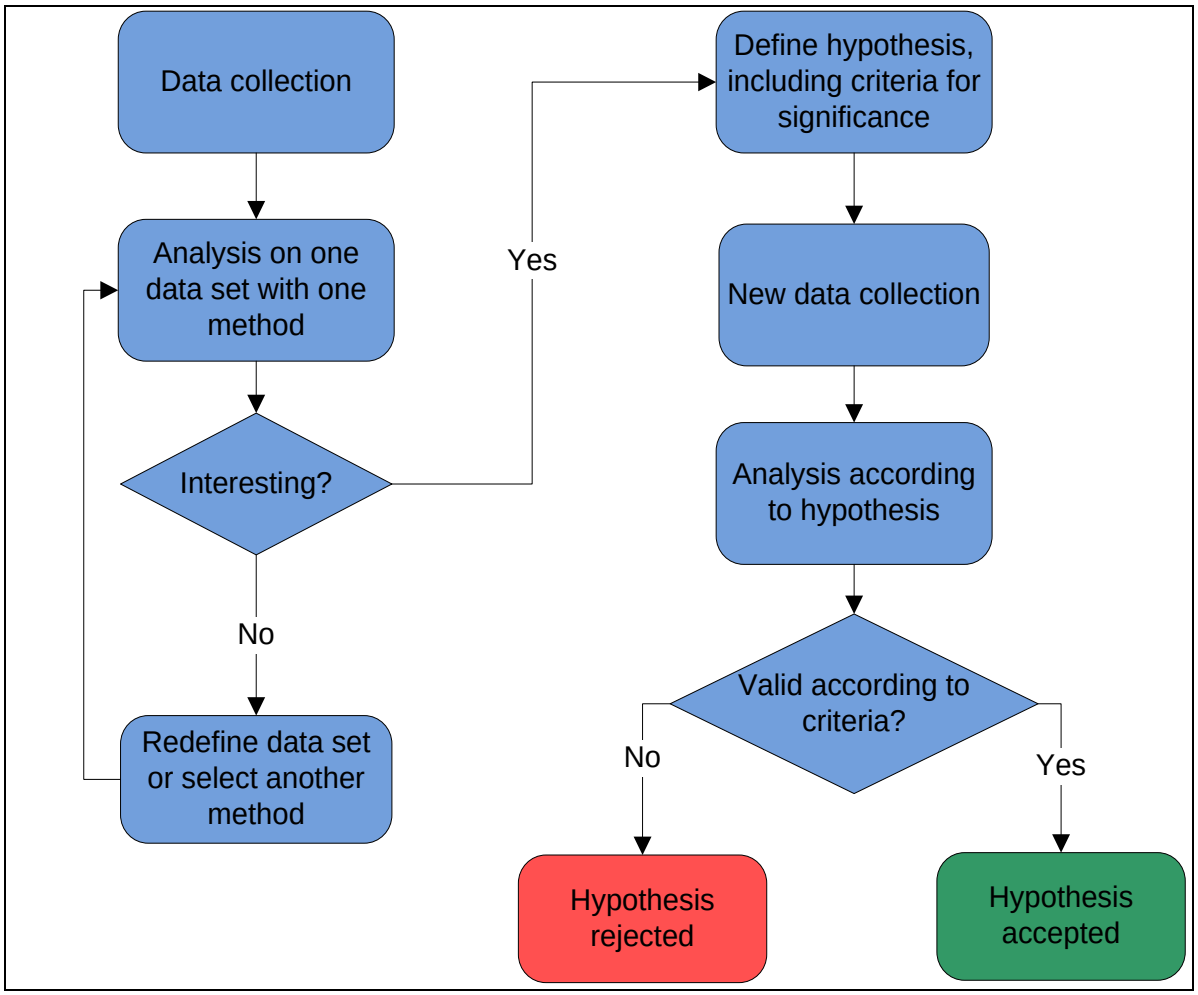


Figure A - 14 Exploratory and confirmatory approaches to statistical studies. This flowchart illustrates the exploratory approach on the left-hand side and the confirmatory approach on the right-hand side.

A.6.3.3 Tools for qPCR data analysis

With the collection of large data sets and demand for more advanced data analysis, software tools for data handling and analysis provide useful support. Dedicated data analysis software provides valuable guidance, although it is no substitute for expert consultations with knowledgeable biostatisticians.

One of the first tools developed for qPCR data analysis was REST (relative expression software tool)[40], which was developed to address the problem of comparing target gene expression levels relative to reference genes in different samples (http://www.gene-quantification.de/rest-2009.html).

Two other commercial software tools offering a high level of power and sophistication are GenEx and qbasePLUS.

GenEx (http://www.multid.se/) provides a vast range of data analysis tools as well as a great deal of flexibility in its data handling. The range of data analysis tools in GenEx includes fundamental data pre-processing, linear regression, t-test, hierarchical clustering and more. For accurate and robust normalisation of qPCR data, GenEx has the advantage of incorporating both NormFinder[41] and geNorm[32] in the software. geNorm is the most popular algorithm and considered the gold standard for reference gene validation. However, the presence of NormFinder in GenEx provides for a convenient cross-validation of the algorithms as well as a validation of the reference genes.

qbasePLUS (http://www.biogazelle.com/qbaseplus) was developed by the inventors of the geNorm[32] algorithm and the qBase[42] quantification model. The software allows in depth quality control, inter-run calibration, normalisation and interpretation. qbasePLUS is MIQE compliant and has extensive data import capabilities for all major qPCR instruments. In addition, it is the only non-web-based software tool that can run natively on Mac, Linux and Windows operating systems.

A.7 Troubleshooting

Following the recommendations that have been provided throughout this guide should result in high quality assays, in most instances. Inevitably, some experiments will fail or result in unexpected data. In many cases the explanation is likely to be operator error since a simple repeat of the experiment often yields the expected results. However before repeating an experiment, a thorough analysis of the data should be made to identify common problems and even where the error may have occurred.

The potential reasons for any assay to yield incorrect data are:

- Operator error – even the most skilled scientists make mistakes now and again;
- Instrument error – this will be most obvious in assays used to analyse several gene targets. Unusual patterns may be noted such that there is a positional relationship between affected sample wells. Sudden spikes in data usually indicate instrument issues;
- Reagent batch failure/incorrect reagents used for the instrument or assay (e.g. SYBR® Green I reagents with a probe assay);
- Template inappropriately checked for quality (i.e. incorrect concentration, inhibitors present, template degraded);
- Probe label may be photobleached – due to poor manufacture or incorrect storage;
- Inappropriate T_m for primers;
- Inappropriate primer concentrations;
- Incorrect or inappropriate sequences (e.g. gDNA or cDNA specific assay used for the wrong template);
- Contamination;
- Inappropriate PCR profile executed for the reagents used, e.g. antibody-inactivated enzymes need very short activation times and are less efficient if longer times are used and vice versa. A chemically modified enzyme must have an adequate activation step or else the assay will fail completely.

For an assay that has not been previously validated there may be additional considerations:

- Poorly designed assay – this will give data but it will be of low quality, resulting in low efficiency, sensitivity and potentially poor specificity;
- The probe labelling or quenching is inadequate – resulting in low fluorescent yield through the reaction;
- The oligonucleotides are incorrect – due to incorrect design, ordering or synthesis mistakes.

Each of these possible causes of failure should be investigated in a logical manner. Some of the most usual problems and troubleshooting steps are described below.

A.7.1 No amplification

An experiment where all reactions have failed completely for all samples is incredibly rare and is usually caused by a catastrophic failure event during the reaction set up or running. Troubleshooting is difficult because any of the potential problems listed above can result in a completely failed reaction. If the reaction has previously functioned correctly, the first step to take is a detailed examination of the reaction protocol, samples used on this occasion, and remaining

reagents. When an obvious fault is not apparent, there are troubleshooting analyses that can be carried out on the failed data file before launching into a series of additional experiments. It can also be useful to differentiate between PCR failure and fluorescent detection failure. However, care must be taken to balance the need to identify exactly which component part of a reaction has failed against completing the experiments in hand. Where reagents are the cause of a problem it often does not matter which one failed and it is economically more viable to replace the entire kit, rather than to spend time identifying specifically whether the *Taq* or the dNTPs have degraded.

A.7.2 Fluorescent detection failure

Although many of the analysis software packages provided with most instruments have a default setting that displays a view of the data after automatic analysis has been performed, there are usually other data display modes that are useful during the troubleshooting process. The raw data (or multi-component plots) show the fluorescent readings throughout the experiment. Different instruments have various views for the data and the user should become familiar with these when analysing reactions with expected results. By repeating this process the range of normal values becomes familiar and it is easy to spot deviations from expected results.

A.7.2.1 How to detect fluorescence failure

Ensure that the instrument is programmed correctly and that data collection occurred at the correct stage of the reaction using the correct wavelength.

View the raw data and compare the background values to those expected (observed previously) when using a probe with the same label/quencher combination, or the same SYBR® Green I reagents with template of a similar concentration.

Significantly lower background values suggest a poorly labelled probe or photobleached SYBR® Green I reagents. When using SYBR® Green I reagents, it is possible that the low background indicates that no template was included in the reaction, although when only low nucleic acid concentrations are included it is difficult to detect SYBR® background in any sample.

When using a probe that is inadequately labelled, the background fluorescence is much lower than expected and there is little or no difference in signal for the label wavelength between the first and last cycle. When using a probe that is inadequately quenched, significantly higher background fluorescence around the upper limit of the range of values for the instrument is observed; again no difference is detected between the start and end of the reaction.

A.7.2.2 How to identify faulty probes

When using a probe for detection, use the same primers in SYBR® Green I reagents (ensure that the correct wavelength is used for detection). A positive reaction confirms that the probe is unsuitable. Check the probe design information in conjunction with the raw data analysis.

A.7.2.3 How to identify weak SYBR® Green I reagents

Repeat the assay using a fresh batch of reagents. Include a positive control with a primer pair that is known to work well.

A.7.3 PCR failure

If no problem can be identified in the detection of the reactions, a simple approach to verify that the PCR step was successful is to use gel electrophoresis. Care must be exercised when performing this analysis since the wells potentially contain high concentration of the gene of interest and therefore pose a serious contamination risk.

A.7.3.1 Failed amplification of some samples

When a single master mix has been used to amplify several samples and while some give amplification, if one or more samples fail to generate a signal, it is clear that there is a problem with the samples or specific reactions.

Examine the plate and/or tubes and ensure that the volume in the failing wells is correct (not reduced due to evaporation), free of bubbles, and that the tubes have not been labelled in a manner to obscure the excitation/detection light path. If no obvious physical reason is evident, consider the possibility that the target may be present at undetectable levels in those samples, or that there may be an inhibitor of the RT and/or PCR step that has carried through into the sample. Inhibitors may be diagnosed by carrying out the SPUD assay (see Section C.2), or by diluting the sample by 10-fold and 100-fold and repeating the assay. Diluting the sample may dilute the inhibitor and hence is potentially a method to rescue an inhibited reaction. Note that inhibitors may affect assays differently and so this may aberrantly affect data analysis relying on ratios of measured genes.

In some cases a reaction will not fail completely, but will generate poor quality data due to low signal amplitude. This is evident in assays with poor efficiency, precision and/or sensitivity. They are often characterised by low fluorescent yield which may be due to poor assay design or probe manufacture.

A.7.4 Unexpected results in controls

Controls should be designed to provide sufficient information about the experiment such that if unexpected results occur there is sufficient information to diagnose the problem (see Appropriate controls, section A.2.4).

The most suitable negative control is a matched sample which has been treated in exactly the same way as the unknown samples and has been verified for absence of all the required targets. In addition a water control (NTC) provides a measure of reaction set up contamination and is an extreme test of the propensity of the primers to create primer-dimer structures. An RT negative control should be included after initial RNA preparation in order to identify samples that contain gDNA contamination.

A negative control with a positive signal indicates that there is contamination at some level of the assay. It could be that the assay is not specific to the desired target (if not previously validated in this control), or that the negative control has become contaminated with target during reaction set up. A parallel water control (NTC) is used to identify reaction set up contamination. While a control which is perfectly negative is preferable, there are situations that may allow for compromise, e.g. a research project measuring cDNA/RNA of a specific transcript which is highly expressed could tolerate small amounts of gDNA target.

When using double-stranded binding dyes (e.g. SYBR® Green I) or specific types of probes such as Molecular Beacons® or Scorpions® as the detection signal, a post-reaction melt curve analysis can be performed. This produces some diagnostic information with regard to the specificity of the amplicon produced, as described previously (see Melt curve analysis, section A.3.5.2). Analysis of the melt profile of the positive control alongside the negative control (giving a positive result) may provide information about the contaminating signal. When using double-stranded binding dyes, a melt point with a lower T_m than the desired amplicon is usually indicative of primer dimerisation, while one higher than the desired cDNA amplicon is normally considered to be derived from gDNA contamination.

In either of these cases, and when multiple peaks are being produced, it is also possible that non-specific hybridisation results in multiple unwanted products. Occasionally a single product can result in multiple melting T_m profiles. This occurs when some of the single-stranded DNA of the amplicon does not anneal precisely resulting in differential melting. Multiple melting profiles that cannot be identified from controls should be further examined by gel electrophoresis.

Section B. Applications of qPCR

The basic simplicity and robustness of the qPCR technique has resulted in its successful application in a wide range of areas, from healthcare and clinical research to food safety and authenticity. The following section discusses specific challenges and considerations for some of the most common applications, such as food authenticity and GMO analysis, pathogen detection and microRNA (miRNA) expression profiling.

B.1 Use of PCR and qPCR in food authenticity studies

Please note that whilst the analytical community involved in Genetically Modified Organism (GMO) analysis at the EU level recognises the importance of standardising measurement responses, a number of methods and approaches in the international guidance still refer to C_t values (cycle threshold values) and have yet to accept and utilise the MIQE guidelines fully. For the purposes of this section, C_t values can be viewed as being equivalent to C_q values in terms of definition.

The DNA molecule is a particularly useful target for food authenticity and food adulteration studies, as it is very resilient to degradation from high temperatures which typically occur during the food processing stages. This potentially enables DNA to be analysed in a range of materials including raw, cooked and processed samples. Compared to using proteins as a target to analyse, the DNA molecule is ubiquitous as it is found throughout all tissues of an organism or food ingredient, whilst proteins can be developmental and tissue specific. The choice of DNA target is only limited by knowledge of the DNA sequence itself, whereas for detection of proteins the availability of antibodies can be a limiting factor. DNA also displays good performance characteristics of specificity and sensitivity, and has the potential to be used in both quantitative as well as traditional qualitative assays. In many respects, approaches that detect DNA as the target can be considered as confirmatory or alternative approaches to those that detect proteins.

The basic and most common approach for DNA analysis is to use PCR. However, the traditional PCR approach is only normally regarded as qualitative – only the presence of absence of the target can be detected. The more advanced approach of qPCR is increasingly being used in food analysis as it permits accurate determination of the initial DNA amount in relative terms.

Whilst the DNA molecule is relatively resilient to degradation in the face of elevated temperatures often encountered during food processing, it is still useful to design assays that amplify DNA fragments of 150bp or less, in order to increase the chances of the target sequence remaining intact.

B.1.1 GMO analysis

Analysis of GMOs is relevant to testing for food authenticity and food adulteration. A GMO is an organism formed from the association of two or more DNA sequences arising from different species, using molecular techniques. Within the EU, and particularly within the UK, public confidence in the use of GMOs is low. Concerns have been raised regarding relevant safety precautions associated with the use of GMOs, for example if there is a chance of transgenic escape of the GM gene from the transgenic crop into a related crop plant or distant plant relative in the environment. One way to address these concerns is to provide the consumer with a choice about whether or not to purchase such GM material. This necessitates a threshold value for labelling of GMOs. Within the EU the traceability and labelling of GMOs is governed by the relevant EU legislation that stipulates products containing GM material must be clearly labelled as containing ingredients derived from GM sources. For EU authorised GMO varieties, food products that contain less than 0.9% GM mass per mass of a particular ingredient, do not need to be labelled as this is thought to be due to adventitious or technically unavoidable contamination.

Being able to detect around the 0.9% mass per mass limit of a particular ingredient is very challenging. Detection at this level therefore often requires trace level analysis of DNA: something that may not be possible if proteins were the target analyte due to inherent issues associated with protein analysis. Successful labelling of food produce is therefore dependent upon a reliable, stringent and efficient way of quantifying GM material.

European legislation on GMOs

EC Regulation No. 1829/2003 lays the foundation for the establishment of a European Union Reference Laboratory for GMOs in Food and Feed (EURL-GMFF), whose core task is the scientific assessment and validation of detection methods for GMOs as part of the official EU authorisation procedure. The legal mandate of European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF) is described in Regulation (EC) No. 1829/2003 and Regulation (EC) No. 882/2004. Further details on the EU Legislation on GMOs can be found at the following site: http://ihcp.jrc.ec.europa.eu/our_activities/gmo/eu-legislation-gmos

B.1.1.1 GMO analysis using qPCR

For unequivocal identification of GMO varieties, it is strongly recommended to use the European Union Reference Laboratory for GMOs in Food and Feed (EURL-GMFF) validated protocols, based on event-specific detection of GMOs using qPCR. These protocols have been subjected to collaborative international inter-laboratory validation studies and include highly prescriptive experimental settings such as PCR conditions, sequence information on primers and probes, and often include details on DNA extraction and optimal DNA concentration. Such protocols have been validated in terms of the performance characteristics of reproducibility, trueness, detection capability (limit of detection and limit of quantification), linear working range (calibration) and specificity. The publicly available database for GMO detection methods should be consulted for the correct PCR or qPCR approach to use: http://gmo-crl.jrc.ec.europa.eu/gmomethods/

Current GM detection strategies using PCR and qPCR may target different genetic elements present in a GMO, and these assays will differ in their specificity accordingly. For example, assays can target the promoter or terminator region involved in a genetic transformation. Such assays are typically low in selectivity as these GM control elements can be found across a range of GM varieties and will not give unequivocal identification of one particular GM event. However, this aspect can also be capitalised upon as the general nature of such an assay lends itself to screening for a range of GM varieties present in the one sample. At the other end of the spectrum, qPCR can be used to target the actual GM event itself: this is the DNA sequence at the site of integration of the genetic modification into the genome of the specific plant species. Such GM events and the integration region that are targeted as part of this approach are unique and specific for each GM variety.

A typical way to quantify the GM content of a test sample is to evaluate the measurement response of this test sample relative to a calibration curve.

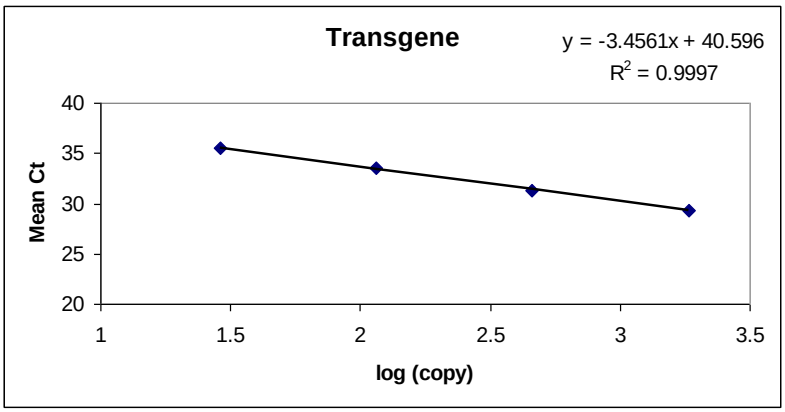
The calibration curve is normally produced by measuring the qPCR measurement response (usually expressed in terms of cycle threshold (C_t) values) from a range of reference materials of known GM content. The accuracy of the measurement may be subject to matrix effects. Matrix effects occur where the matrix from which the DNA has been extracted for the calibrators and test sample differ, and this can impact upon PCR efficiency through the co-extraction of PCR inhibitors. It is possible to overcome such concerns by using alternative approaches, such as digital PCR, that offer the potential for absolute single molecule detection, but these techniques require further validation before they can be qualified for routine GMO quantification purposes.

It is advisable that the reference materials used to construct the calibration curve are of the highest possible quality, and this criterion can be met most easily by using Certified Reference Materials (CRMs).

B.1.1.2 Calibration curve approaches for GMO analysis

There are a number of different approaches that can be used to construct calibration curves for GMO analysis, and the data needs to be treated appropriately to ensure accurate interpretation.

Serial dilution series from a single CRM, certified on the basis of m/m, can be used to construct separate calibration curves for both the transgene (GM trait) and endogenous (taxon specific) targets. These calibration curves are often constructed by plotting the logarithm of the copy number of the CRM against the C_t value (qPCR measurement response) (see Figure B - 1). The test sample is then evaluated for its transgenic and endogenous responses relative to the two calibration curves, and the GM content of the test sample estimated by expressing the number of transgene copies relative to the endogenous copies. The measurement unit associated with the result remains the same as the measurement unit of the calibrator: that of percentage GM expressed in terms of m/m.



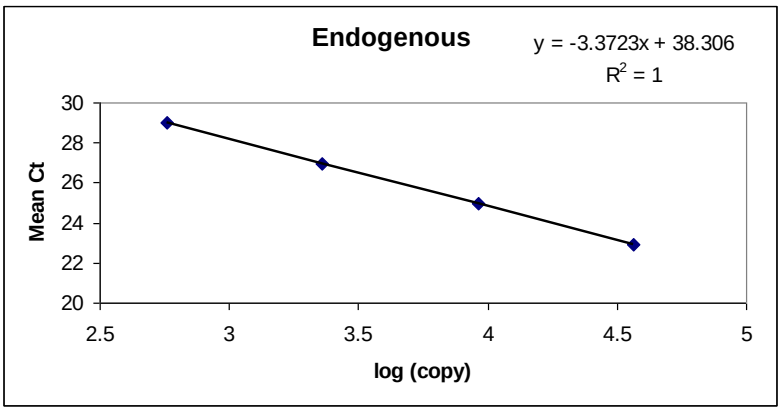


Figure B - 1 Separate transgene and endogenous calibration curves based on serial dilution series of a CRM.

A second approach is to produce just one combined calibration curve in terms of what is commonly referred to as the "delta C_t approach". This uses a range of CRMs that differ in their GM content. The qPCR measurement response from the transgene and endogenous targets are measured for each CRM and expressed as delta C_t according to the following equation (6):

$$\text{Delta } (\Delta)\ C_t = (\text{Transgene } C_t) - (\text{Endogenous } C_t) \qquad (6)$$

The calibration curve is typically constructed by plotting the logarithm of the %GM content of the CRMs against the delta C_t value (see Figure B - 2). As before the test sample is evaluated in terms of its transgene and endogenous C_t responses, which are then transformed into a delta C_t value in order to estimate the GM content.

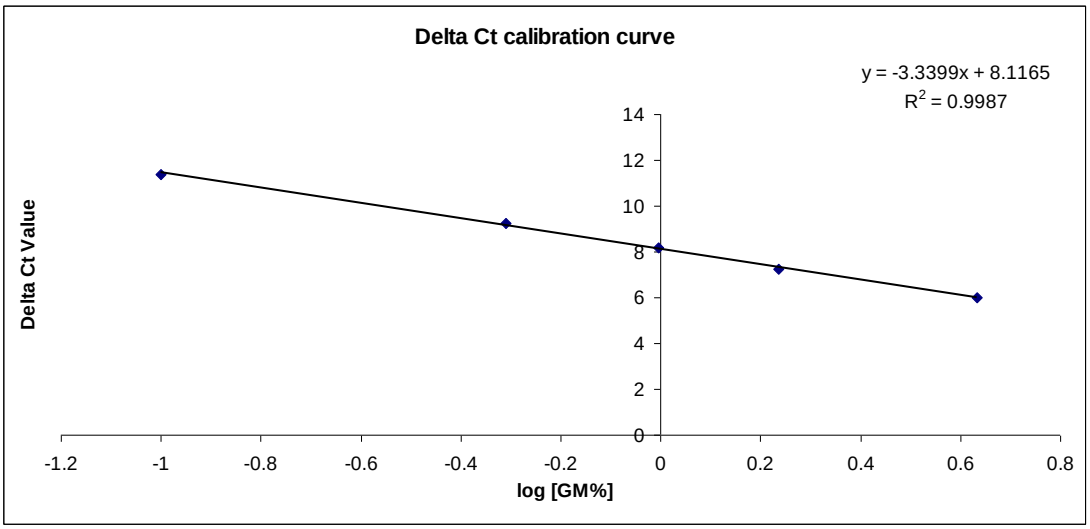


Figure B - 2 Delta C_t calibration curve.

The third and final calibration curve approach is based on using a plasmid calibrator certified for its copy number ratio, and expressing results in terms of copy numbers. A dilution series of the plasmid CRM is prepared and the transgene and endogenous qPCR measurement responses recorded. The calibration curve is typically constructed by plotting the logarithm of the copy number against the qPCR measurement response (C_t) for both the transgene and endogenous assays, resulting in two calibration curves (see Figure B - 3). The test sample is measured for its transgene and endogenous responses, these are then transformed into the relative copy numbers using the relevant calibration curves, and the GM content of the test sample expressed in terms of copy numbers based on the transgene copies relative to endogenous copies.

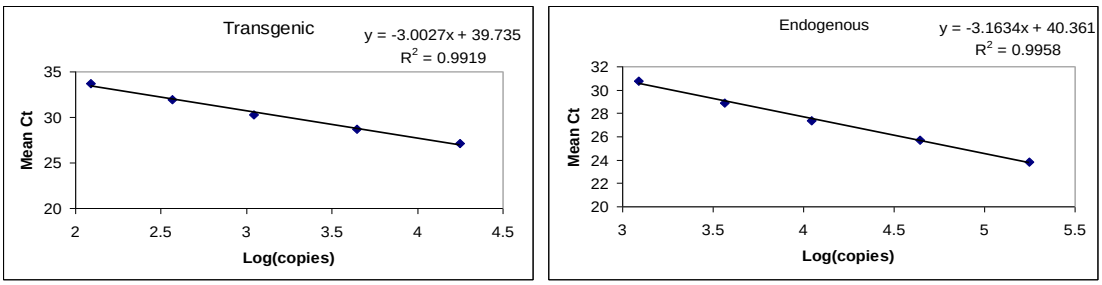


Figure B - 3 Separate transgene and endogenous calibration curves based on a serial dilution series of a CRM plasmid calibrator.

B.1.1.3 GMO screening approaches

As described above, it is advisable to use the EURL validated protocols for event-specific detection and quantification of specific GM varieties. However, the current influx of unauthorised GMOs into the EU often precludes practical application of each event-specific protocol as this is not cost effective, and a more general screening approach may be required. The EU is currently co-ordinating a standardised approach for developing a method for GMO screening between EU member countries, including the use of bio-informatics tools to help maximise the confidence of identifying the correct GM variety.

There are a number of approaches available for GMO screening using PCR, many of which are based on commercially available kits or offered through a commercial service provider. One such approach is to use the “Real-Time PCR-Based Ready-to-Use Multi-Target Analytical System for GMO Detection”, which permits the simultaneous screening of 39 GMO varieties in one

experimental run/batch.[43] This is based on real-time PCR and uses a 96 well microtitre plate that contains lyophilised primers and probes. This permits event-specific simultaneous detection of 39 GMO varieties and provides a rapid and cost-effective assay for screening for 39 GM events as well as common crop reference genes. Its use is currently restricted to EU Official Control Laboratories but the EURL-GMFF plan is to make this available to the wider analytical community in the future.

An alternative approach for GMO screening is offered by the "GMO multiplex screen" protocol.[44] This approach was originally developed by Campden BRI as part of a UK Food Standards Agency project (G03022) and relies upon standard end-point PCR and capillary electrophoresis. The approach uses a qualitative multiplex end-point PCR approach for the simultaneous detection of GM soya, GM maize, and the common GM control elements of P-35S (promoter sequence) and TNOS (terminator sequence). The protocol provides a simple, cost effective approach for screening food ingredients for GMOs in raw materials and some processed foods. The approach was originally optimised and validated using the Agilent Bioanalyzer 2100 but LGC was able to validate its applicability across further laboratory instrumentation that utilise capillary systems, including the Lab901 TapeStation and the Shimadzu MultiNA. The results of this validation exercise showed that each of the three alternative instruments had its own advantages in terms of bias, precision and throughput, but all three instruments gave correct identification of all samples tested. As alternative instruments, all three are comparable and fit for purpose, providing greater scope for DNA food analysis.[19]

B.1.2 Sample verification (using meat and pasta examples)

Whilst qPCR is the analytical method of choice for the detection and quantification of GMOs at European level, its use for food authenticity purposes is also extended to a range of other examples, two of which will be briefly considered here.

B.1.2.1 Meat authenticity

There are a number of commercially available kits and approaches described in the published literature that use qPCR for the identification of DNA arising from meat species (predominantly lamb, pork, beef and chicken). A common issue encountered with some of the published techniques is that many of the qPCR approaches for meat speciation tend to demonstrate cross-reactivity during later cycles of the PCR. This is shown by false positives occurring when a species-specific primer and probe assay amplifies non-target DNA from other meat species after around 35 cycles. Previous work in this area[45] found species identification was possible by limiting the PCR to 30 cycles, which effectively removed assay cross-reactivity seen during the later cycles. In this publication the authors noted cross reactivity associated with assays above 30 cycles of the PCR, and so introduced a "C_t cut off value" above which any measurement response was considered as a false positive.

This approach used just two probes to differentiate the five key species (beef, pork, lamb, chicken and turkey); one universal for mammalian species and one universal for poultry. The C_t values they achieved, based on normalising the DNA content to 50 ng of template per reaction, were between 17 and 18 cycles for all species except chicken, which produced C_t values in the range of 20-21. Whilst this approach may be useful in some cases, it does convey the risk of incorrectly rejecting a true positive: for example if the sample had a very low amount of the correct target species present or if the DNA from a processed food sample had been degraded.

In order to increase selectivity and decrease the likelihood of false positives or false negatives occurring, an alternative approach of using melt curve analysis can also be conducted. (See section A.3.5.2 on Melt Curve analysis).

B.1.2.2 Pasta authenticity

Pasta must be made from *Triticum durum* (Durum wheat), which is a low yielding but high quality variety of wheat. Current EU law permits up to 3% adventitious contamination of pasta with other

wheat (*T. aestivum* or common wheat), but gross contamination results in loss of quality and cooking characteristics of the pasta, and also impacts upon legitimate businesses and UK trade.

A method has been devised for the quantitative analysis of common wheat adulteration in commercial dried pastas using qPCR. This measures the relative amount of DNA from the D-genome of wheat (indicating presence of adulteration with common wheat *T. aestivum*)[46] in relation to a normalisation sequence found in all wheat varieties. A single delta C_t calibration curve was used based on plotting the logarithm of the percentage adulteration associated with a range of standards, against the C_t value. In order to increase the applicability of this approach to processed food samples the PCR products were designed to be around 120bp or less. Quantification of the DNA template prior to use within PCR was also found to be a critical factor in the quantitative capability of the approach, and normalising the starting amount of DNA to 50-100 ng per PCR was advocated.

B.1.3 Summary to food authenticity testing using real-time PCR

Current EU legislation for food labelling has a number of stakeholders including food manufacturers, retailers and traders, and enforcers of legislation. All these stakeholders require access to accurate methods to determine the level of different materials and ingredients present in foodstuffs. DNA is often used as the target analyte in such tests for food authenticity and adulteration, with PCR and qPCR providing the analytical framework for such approaches. There are a variety of different PCR methods, instruments and approaches that are used in food authenticity analysis, and these all need to be fully validated to ensure confidence in interpretation of a result. Part of the method validation process will involve characterising the measurement uncertainty associated with the results from the analytical approach. This will help standardise results at a national and international level, and provide objective evidence of the fitness for purpose of a particular analytical approach.

B.2 Pathogen detection for clinical analysis

At present, the main methods for pathogen detection are well-established, and these include culturing and microscopy. However, serological tests and, more recently, molecular methods targeting pathogen nucleic acids have provided a valuable complement to existing approaches.

It is worth noting that the application of molecular methods to the identification and management of infectious diseases has been most successful where no other method is readily available, e.g. for viral monitoring and resistance analysis. Where they have been applied in areas such as bacterial identification in sexually transmitted infections, there has been a clear considerable advantage over existing methods because viable organisms are not needed for molecular methods and thus the diagnostic sample is more stable.

Designing and performing PCR-based detection methods for pathogens that can be used clinically has a number of additional considerations. This is because the findings can directly affect patient prognosis but also there may be additional epidemiological considerations that need to be taken into account when interpreting a result. Analytical sensitivity and specificity are both important factors that need defining.

Analytical sensitivity - the ability of the qPCR technique to detect low numbers of copies - is concerned with the limit of detection and quantification. It will be influenced by the assay design, optimisation and choice of reagents, but with currently available reagents and published protocols, this limit of detection can usually be down to near single copy.

Analytical specificity – the ability of the technique to detect the target of interest in the presence of other components – is primarily influenced by assay design and optimisation. To be specific for a particular organism, assays must be designed to target unique sequences and not regions of conservation between related species. Assay optimisation can also be important as poorly optimised assays may amplify non-specific targets yielding a positive signal even when the target DNA of the organism is not present.

The analytical criteria described above are not limited to pathogen diagnostics and can apply to many aspects of PCR. However, the clinical applications of pathogen detection add a level of complexity to the experimental design. Diagnostic sensitivity, like analytical sensitivity, is concerned with detecting the presence of the pathogen under investigation. In addition to considerations regarding analytical sensitivity, diagnostic sensitivity also addresses specific disease factors such as whether the diagnostic analyte is constitutively present for a given disease. Diagnostic sensitivity can be compromised by the heterogeneous nature of the sample or by the presence of a low number of pathogens. If the result is negative, it will be difficult to determine whether undetectable small numbers of a pathogen can cause the disease, or if the pathogen is simply not present.

Diagnostic specificity can be another major challenge as the analytical specificity of a technique may be perfectly capable of detecting a potential pathogen. However, if the pathogen is present in the test sample but not responsible for the observed symptoms, it may be incorrectly identified as the cause.

Additionally, diagnostic tests must also consider other epidemiological factors of the disease in question. Particularly, the prevalence (proportion of population with the condition) of a disease directly influences the popularity of a particular test. When the disease prevalence is low, specificity becomes a crucial technical consideration. When more negative patients are tested than positive patients, the incidence of false positives increases.

The use of qPCR to quantify the amount of a pathogen, rather than for qualitative detection (of the presence of the pathogen) or for sequencing purposes, remains limited to a small number of virological targets that includes HIV, hepatitis C and B and Cytomegalovirus. Viral load is measured by qPCR or RT-qPCR as a prognostic indicator to support patient management. One

of the challenges associated with viral load measurement is ensuring that different laboratories report comparable results, which may be improved by using reference materials.[47]

Quantification of other pathogens may also have a valuable role and there is evidence that monitoring tuberculosis treatment may provide a good prognostic indicator.[48] A qPCR-based method has been developed by Cephaid to diagnose pathogens including tuberculosis.[49] Work will be required to establish the impact of these methods on geographic regions where the burden of the disease is highest[50], and approaches for ensuring quality control will be crucial. However, the research in this field is very promising and qPCR has a huge potential to improve the diagnosis and management of infectious diseases.

B.3 MicroRNA expression profiling

MicroRNAs (miRNAs) are short (~20 nucleotides), non-coding RNA molecules that play essential roles in diverse biological processes, including cell fate determination, cell proliferation, cell differentiation and cell death.[51,52] They are post-transcriptional regulators of gene expression that function by interacting with and downregulating messenger RNA (mRNA) .[51, 52] Since the recent discovery of miRNAs in mammals, there has been an explosion of research activity aimed towards understanding their biological functions. There has also been a growing interest in their use for clinical applications.[53,54]

Over the past few years, RT-qPCR has become one of the most popular methods for profiling miRNA expression. However, miRNAs cannot be amplified using the same methods as larger RNA species and there are several challenges associated with the detection of miRNAs that RT-qPCR technologies must overcome:

- The small size of miRNAs is a huge technical challenge to the design of a successful priming strategy;
- miRNAs are heterogeneous in their GC content making it difficult to design primers with common annealing temperatures across all targets within a population;
- Specificity is a major issue affecting miRNA detection as miRNAs can exist in families where sometimes only one or two nucleotides differ between family members;
- miRNAs are processed from precursor molecules that contain the same sequence as the fully processed mature miRNA and qPCR technologies must be able to distinguish between them;
- miRNAs lack a common feature, such as a poly(A) tail, that would facilitate their selective purification or reverse transcription by a universal probe.

However, despite these pitfalls, several highly effective miRNA RT-qPCR assays have recently been developed and are summarised in Table B - 1. The Taqman® miRNA Assay from Life Technologies is currently the most widely used assay, partly because it is very well established, being one of the first such assays to reach the marketplace.

The Taqman® miRNA assay employs a miRNA-specific stem-loop reverse transcription (RT) primer to generate cDNA for subsequent qPCR amplification using dual-labelled hydrolysis probes (see Figure B - 4). The structure of the stem-loop RT probe reduces binding to precursor molecules and potential contaminating gDNA by steric hindrance, thereby increasing the specificity of detection of the mature miRNA. This feature of the stem-loop priming method provides a distinct advantage over methods which use linear RT primers. The combination of a miRNA-specific RT step with hydrolysis probe-based qPCR results in highly specific miRNA amplification.

In contrast, the other miRNA assays use linear universal RT primers combined with SYBR® Green (or EvaGreen®) technology to amplify the target miRNA, with the exception of the mirVana™ qRT-PCR miRNA Detection Kit which uses linear miRNA-specific RT primers. For the majority of SYBR® Green-based assays, the addition of a poly(A) tail to mature miRNA is achieved using the poly(A) polymerase enzyme, allowing the subsequent binding of an oligo(dT) containing universal primer. The universal RT primer enables the same cDNA preparations to be used for multiple miRNA assays, a particular advantage when limited starting material is available.

Multiplexed miRNA RT-qPCR assays are also available for hydrolysis probe based methods. The Exiqon miRCury LNA™ Universal RT PCR (see Figure B - 4) assay is one of the most prominent miRNA RT-qPCR SYBR® Green methods and is distinct from the others in that it uses Locked Nucleic Acid (LNA) containing qPCR primers. LNAs (see section Primer and probe design, A.3.3) are chemically modified RNA molecules that significantly increase the hybridisation

properties of DNA oligonucleotides, affording higher melting temperatures for primers which bind to relatively short regions of template. This gives rise to effective discrimination between closely related miRNAs with few nucleotide differences.[55] Other miRNA assays, which are solely DNA primer based, also report good discrimination between miRNA family members. A recent report has also suggested that LNA containing DNA templates are not amplified with a high efficiency.[56] A general advantage of using SYBR® Green based assays, which should also be considered, is that the specificity of the assay can be tested by performing melting curve analysis.

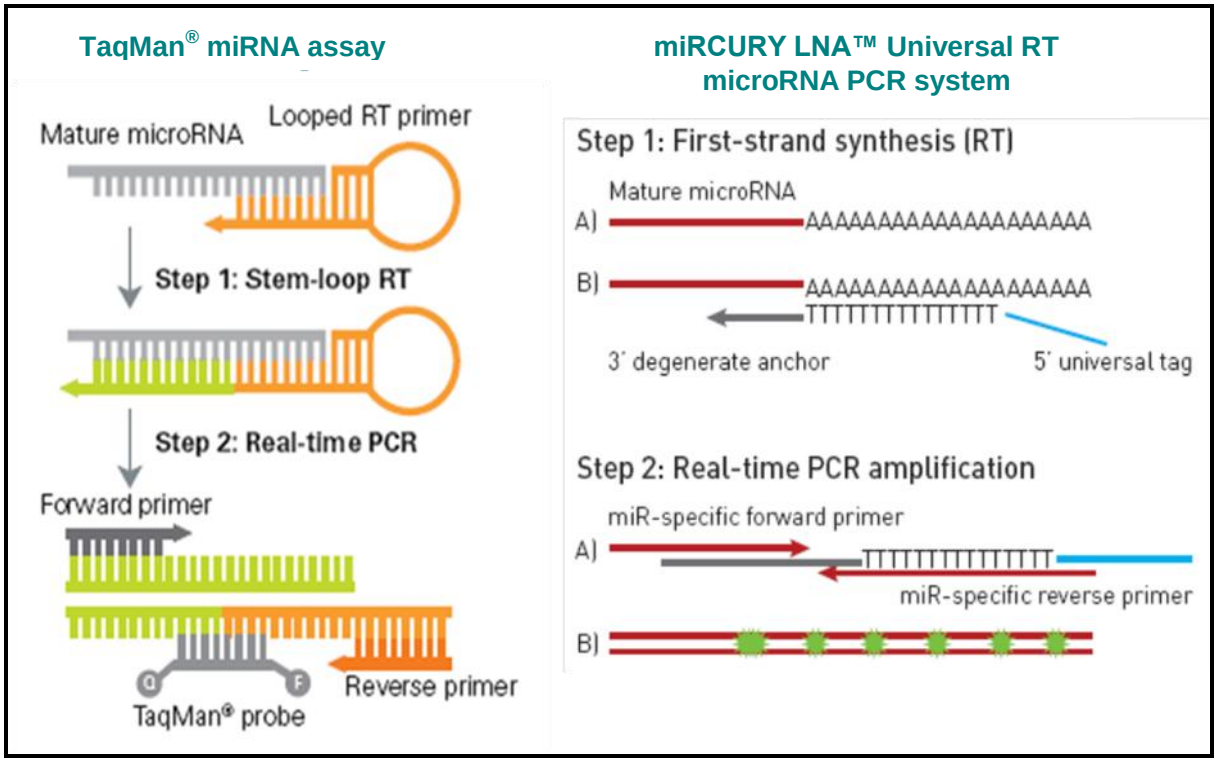


Figure B - 4 Illustration of TaqMan® miRNA assay from Life Technologies[57] and the miRCURY LNA™ Universal RT microRNA PCR System from Exiqon (reproduced with permission from miRCURY LNA™ Universal RT microRNA PCR system manual V 5.2, March 2013, © Exiqon).

Scientists at the European Molecular Biology Laboratory (EMBL) have devised an alternative method of profiling microRNA expression using quantitative real-time PCR. The method called miQPCR exploits the activity of T4 RNA Ligase 1 to covalently attach the 3'-hydroxyl group of mature miRNAs to the 5'-phosphate group of an RNA/DNA linker adaptor, which comprises a universal primer-binding sequence. The extended miRNAs are then transcribed using a universal primer complementary to the 3'-end of the linker. Assay specificity and sensitivity are determined at the qPCR stage with the use of miRNA specific primers while detection of qPCR products is achieved by using SYBR Green.

Researchers embarking upon miRNA studies for the first time should carefully consider the advantages and disadvantages of each assay before deciding which is the most suitable for the research project in question.

Company	Assay	RT primer type	qPCR primers	Detection method	References
Life Tech (Applied Biosystems)	Taqman® miRNA assay	Stem-loop	DNA	Dual-labelled hydrolysis probes	Chen *et al* [57]
Exiqon	miRCURY LNA™ Universal RT microRNA PCR System	Linear	LNA-DNA	SYBR® Green	http://www.exiqon.com
Life Tech (Ambion)	mirVana™ qRT-PCR miRNA Detection Kit	Linear	DNA	SYBR® Green	https://www.lifetechnologies.com
Life Tech (Ambion)	NCode™ EXPRESS SYBR® GreenER™ miRNA qRT-PCR Kit Universal	Linear	DNA	SYBR® Green	https://www.lifetechnologies.com
Qiagen	miScript SYBR® Green PCR Kit	Linear	DNA	SYBR® Green	http://www.qiagen.com
Agilent	miRNA qRT-PCR Detection	Linear	DNA	EvaGreen®	http://genomics.agilent.com
EMBL	miQPCR	Linear	DNA	EvaGreen®/ SYBR® Green	Benes & Castoldi Methods 2010 [58] Castoldi et al, JCI 2011 [59]

Table B - 1 Summary of the commercially available miRNA RT-qPCR technologies.

Section C: Protocols

The aim of this guide is to describe the best practice principles underpinning analytically sound qPCR studies. In this section, there are examples of basic qPCR protocols that can implement those principles. They will, hopefully, provide a good practical basis from which qPCR studies can be designed.

Note: The following protocols have been provided by Sigma-Aldrich® and therefore have been validated for the specific Sigma-Aldrich® reagents stated. They do provide a good basis for use with other reagents and systems, however method optimisation prior to sample analysis is strongly recommended.

Protocol index:

C.1 Basic qPCR protocol

C.1.1 Experiment objective

The following protocol may be used as a basic template for modification, or for a quick evaluation of the suitability of a set of primers. In these reactions, primers are included at a final concentration of 450 nM. The reactions are performed using either SYBR® Green I master mix or a probe is included into a qPCR master mix at 200 nM.

C.1.2 Equipment

- Quantitative PCR machine

C.1.3 Reagents

- cDNA diluted 1:10 or gDNA
- KiCqStart SYBR® Green ReadyMix™ (Sigma KCQS00/KCQS01/KCQS02/KCQS03 – depending on instrument) or LuminoCt qPCR ReadyMix
- PCR grade water
- Forward and reverse primers for test gene (stock at 100 µM)
- Probe (for single probe reaction)

C.1.4 Supplies

- Laminar flow hood for PCR set up (optional)
- 1.5 mL tubes, sterile
- Tube racks for 1.5 mL tubes
- 0.2 mL PCR tubes or 96 well plate
- Caps for PCR tubes or plate seal for 96 well plate
- Pipettes
- Aerosol-barrier pipette tips

C.1.5 Safety

- Lab coats
- Gloves
- Eye protection (safety glasses)

C.1.6 Procedure

Notes for these protocols:

- cDNA is generated, for example, using random priming or oligo-dT priming method (as seen in protocol C.4) and diluted 10-fold prior to use in the qPCR reaction;
- 10 µM working solution of primers are prepared from 100 µM stock solutions;
- All reactions are performed in duplicate for technical replication;
- If using a PCR plate, follow a plate schematic to ensure that the reaction mix, samples and controls are added to the right wells.

C.1.6.1 SYBR® Green I reaction

1. Prepare a master mix for all reactions including the No Template Controls (NTC) in duplicate, according to table below. Calculate volumes for each reaction and add 10% to allow for pipetting error. Mix well, avoiding bubbles.

Reactions	Volume per single 20 µL reaction (µL)
2x KiCqStart SYBR® Green qPCR ReadyMix	10
Forward primer (10 µM working solution)	0.9
Reverse primer (10 µM working solution)	0.9
PCR grade water	4.2

2. Transfer 32 µL of master mix from step #1 into a separate tube for setting up the NTC reactions.
3. Add 8 µL of water to the NTC master mix from step #2. Set NTC master mix on ice.
4. Aliquot 16 µL of template master mix remaining from step #1 into each PCR tube or plate well.
5. Add 4 µL of cDNA solution to all the tubes/wells in step #4 except for the NTC tubes/wells.
6. Aliquot 20 µL of NTC master mix from step #3 into the NTC PCR tubes/wells.
7. Cap tubes or seal the PCR plate and label. **(Make sure that the labelling does not obscure instrument excitation/detection light path.)**
8. Process samples according to the protocol below (note these conditions are specific for FAST cycling protocols).

Note: Use standard dissociation curve protocol (data collection)

FAST cycling conditions	Temp (ºC)	Time (s)
Step 1	95	30
Step 2 (40 cycles)	95	5
	58	15
	72	10

C.1.6.2 Single probe reaction

1. Prepare a master mix for all reactions in duplicate, including the No Template Controls (NTC), according to table below (again, incorporating an additional 10% volume to allow for pipetting error). Mix well, avoiding bubbles.

Reaction	Volume per single 20 µL reaction (µL)
2x LuminoCt qPCR ReadyMix	10
Forward primer (10 µM working solution)	0.4
Reverse primer (10 µM working solution)	0.4
Probe (10 µM working solution)	0.4
PCR grade water	4.8

2. Transfer 32 µL of master mix from step #1 into a separate tube for setting up the NTC reactions.
3. Add 8 µL of water to the NTC master mix from step #2. Set NTC master mix on ice.
4. Aliquot 16 µL template master mix remaining from step #1 into the PCR plates or tubes.
5. Add 4 µL of cDNA solution to all the tubes or wells in step #4 except for the NTC tubes/wells
6. Aliquot 20 µL NTC master mix from step #3 into the NTC PCR plate or tubes.
7. Cap tubes or seal the PCR plate and label. **(Make sure that the labelling does not obscure instrument excitation/detection light path.)**
8. Process samples according to the protocol below (note these conditions are specific for FAST cycling protocols).

FAST cycling conditions	Temp (°C)	Time (s)
Step 1	95	30
Step 2 (40 cycles)	95	5
	60	30

C.2 The SPUD assay for detection of assay inhibitors

C.2.1 Experiment objective

The SPUD assay is a first step towards identifying inhibitors present in RNA or DNA samples. The assay is particularly useful when analysing a large number of samples, to avoid false negatives.

The SPUD assay consists of an artificial template and two primers specific to the template and is run in the presence of SYBR® Green I dye or a specific probe. During the SPUD assay the template is amplified, resulting in a characteristic C_q. Alongside this control reaction, the artificial template is spiked into samples and measured in comparison to the control. In the presence of a clean sample the C_q will remain the same as the control, whereas in the presence of a contaminated sample the C_q will shift to higher cycles.

C.2.2 Equipment

- Quantitative PCR machine

C.2.3 Reagents

- cDNA (sample for inhibitor test) diluted as for use in qPCR (usually 1:10)
- KiCqStart SYBR® Green I ReadyMix™ (KCQS00, KCQS01, KCQS02, KCQS03 – depending on instrument)
- PCR grade water
- Forward and Reverse primers for SPUD assay (stock at 100 µM)
- SPUD template oligo diluted to approximately 20,000 copies/µL

Primer	Sequence (5'-3')
Spud Template	AACTTGGCTTTAATGGACCTCCAATTTTGAGTGTGCACAAGCTATGGAACAC CACGTAAGACATAAAACGGCCACATATGGTGCCATGTAAGGATGAATGT
Spud Forward	AACTTGGCTTTAATGGACCTCCA
Spud Reverse	ACATTCATCCTTACATGGCACCA

C.2.4 Supplies

- Laminar flow hood for PCR set up (optional)
- 1.5 mL tubes, sterile
- Tube racks for 1.5 mL tubes
- 0.2 mL PCR tubes or 96 well plate
- Caps for PCR tubes or plate seal for 96 well plate
- Pipettes
- Aerosol-barrier pipette tips

C.2.5 Safety

- Lab coats

- Gloves
- Eye protection (safety glasses)

C.2.6 Procedure

1. Prepare a reaction for each sample and run each in duplicate.
2. Prepare a reaction containing SPUD template but no test sample (NTC), in duplicate.
3. Prepare 10 µM working solution of SPUD primers from 100 µM stock solutions.
4. Prepare master mix according to table below with an additional 10% volume to allow for pipetting error. Mix well.

Reagents	Volume per single 25 µL reaction (µL)
2x KiCq Start Mastermix	12.5
SPUD Primer F (10 µM working solution)	0.25
SPUD Primer R (10 µM working solution)	0.25
SPUD template (pre diluted to 20,000 copies)	1
Reference dye (optional)	1
PCR grade water	5

5. Aliquot 20 µL of qPCR master mix into the sample tubes/wells. If using a PCR plate, follow a plate schematic to ensure that the reagents, samples and controls are added to the correct wells.
6. Aliquot 5 µL cDNA sample into qPCR tubes/wells and 5 µL water to the NTC tubes/wells.
7. Cap tubes or seal the PCR plate and label. **(Make sure that the labelling does not obscure instrument excitation/detection light path.)**
8. Process samples using the protocol provided below.

Note: use standard dissociation curve protocol (data collection)

Cycling conditions	Temp (°C)	Time (s)
Step 1	95	30
Step 2 (40 cycles)	95	5
	60	20

C.3 3’/5’ Assay for analysis of template integrity

C.3.1 Experiment objective

The 3’/5’ integrity assay is a first step in the identification of RNA degradation. The assay is particularly useful for the analysis of a large number of samples.

A target RNA sequence is selected, GAPDH in this example, and two assays are designed along the length of the target such that one is located close to the 5’ UTR and the second is approximately 1 kb upstream. cDNA is generated using reverse transcription from an anchored oligo-dT primer. Following amplification by qPCR, the product from each assay is quantified and the ratio of the quantities is compared to the ratio derived from a sample of high quality cDNA. Degradation of the template results in a relative decrease in product, especially with assays near the 5’ end of the target gene. This results in an increase in the 3’/5’ ratio.

C.3.2 Equipment

- Quantitative PCR machine

C.3.3 Reagents

- cDNA prepared using oligo-dT priming
- LuminoCt™ ReadyMix™ for Quantitative PCR (L6669)
- PCR grade water
- 3’ and 5’ Forward and Reverse primers for GAPDH at 100 µM
- 3’ and 5’ Probes at 100 µM

	Primer	Sequence
5' 3'	GAPDH 5' Forward	GTGAACCATGAGAAGTATGACAAC
5' 3'	GAPDH 5' Reverse	CATGAGTCCTTCCACGATACC
5' 3'	GAPDH 3' Forward	AGTCCCTGCCACACTCAG
5' 3'	GAPDH 3' Reverse	TACTTTATTGATGGTACATGACAAGG
5' 3'	GAPDH 5' Probe	[FAM]CCTCAAGATCATCAGCAATGCCTCCTG[BHQ1]
5' 3'	GAPDH 3' Probe	[Joe]CCCACCACACTGAATCTCCCCTCCT[BHQ1]

C.3.4 Supplies

- Laminar flow hood for PCR set up (optional)
- 1.5 mL tubes, sterile
- Tube racks for 1.5 mL tubes
- 0.2 mL PCR tubes or 96 well plate
- Caps for PCR tubes or plate seal for 96 well plate
- Pipettes
- Aerosol-barrier pipette tips

C.3.5 Safety

- Lab coats
- Gloves
- Eye protection (safety glasses)

C.3.6 Procedure

1. Prepare two reactions per sample for testing and two No Template Controls (NTC).
2. Prepare a 50 µM working solution of GAPDH primers and 10 µM for probes from 100 µM stock solutions.
3. Prepare master mix according to table below, calculating the requirements for the number of reactions and an additional 10% volume to allow for pipetting error. Be sure to mix well.

Reagents	Volume per single 25 µL reaction (µL)
2x LuminoCt Mastermix	12.5
Primer 3' F (300 nM final, 50 µM working solution)	0.15
Primer 3' R (300 nM final, 50 µM working solution)	0.15
Primer 5' F (300 nM final, 50 µM working solution)	0.15
Primer 5' R (300 nM final, 50 µM working solution)	0.15
Probe 3' (200 nM final, 10 µM working solution)	0.5
Probe 5' (200 nM final, 10 µM working solution)	0.5
Reference dye (optional)	1.0
PCR grade water	4.9

4. Aliquot 20 µL of qPCR master mix into the PCR tubes/wells. If using a PCR plate, follow a plate schematic to ensure that the reagents and controls are added to the intended wells.
5. Add 5 µL cDNA sample into qPCR tubes/wells.
6. Cap tubes or seal the PCR plate and label. **(Make sure that the labelling does not obscure instrument excitation/detection light path.)**
7. Samples should be processed using the protocol provided below.

Cycling conditions	Temp (°C)	Time (sec)
Step 1	95	30
Step 2 (40 cycles)	95	5
	55	15
	72	10

C.4 Reverse transcription (one-step and two-step) protocols

Reverse transcription (RT) is the process of converting RNA to cDNA, using a reverse transcription enzyme and dNTPs, prior to analysing the cDNA in the qPCR step.

The RT step may be performed on total RNA such that a global cDNA representation of many transcripts is produced (usually via a two-step protocol), or, in a gene specific approach, only the RNA of interest is converted to cDNA (usually following a one-step protocol).

The following experiments can be used as basic RT protocols that can be modified to suit particular requirements.

C.4.1 Standard reverse transcription protocol (two-step)

C.4.1.1 Equipment

- Standard PCR instrument or heating block

C.4.1.2 Reagents

- RNA (1 µg/µL)
- Readyscript two-step cDNA synthesis kit (Sigma RDRT)
- PCR grade water

C.4.1.3 Supplies

- Laminar flow hood for RT set up (optional)
- 1.5 mL tubes, sterile
- Tube racks for 1.5 mL tubes
- 0.2 mL PCR tubes or 96 well plate
- Caps for PCR tubes or plate seal for 96 well plate
- Pipettes
- Aerosol-barrier pipette tips

C.4.1.4 Safety

- Lab coats
- Gloves
- Eye protection (safety glasses)

C.4.1.5 Procedure

1. Combine reagents in 0.2 mL micro-tubes or 96-well plate sitting on ice according to the table below. If using a PCR plate, follow a plate schematic to ensure that the reagents are added to the correct wells.

Reagents	Volume per single 20 µL reaction (µL)
ReadyScript cDNA Synthesis Mix (5x RT blend)	4
total RNA template – variable (1 µg to 10 pg)	1
PCR grade water	15
Total Volume	20

2. After sealing each reaction, vortex gently to mix contents. Centrifuge briefly to collect components at the bottom of the reaction tube.

3. Incubate reaction mix as follows:

Temperature (°C)	Time (mins)
25	5
42	30
85	5
4	Hold

4. After completion of cDNA synthesis, use 1:5 to 1:10 of the first-strand reaction (2-4 µL) for PCR amplification.

If desired, cDNA product may be diluted with 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA and stored at -20 °C.

C.4.2 Standard reverse transcription protocol (One-step SYBR® Green)

C.4.2.1 Equipment

- Real-time PCR instrument

C.4.2.2 Reagents

- RNA (1 µg/µL)
- SYBR® Green Quantitative RT-PCR kit (Sigma QR0100)
- PCR grade water
- Primers

C.4.2.3 Supplies

- Laminar flow hood for RT-qPCR set up (optional)
- 1.5 mL tubes, sterile
- Tube racks for 1.5 mL tubes
- 0.2 mL PCR tubes or 96 well plate
- Caps for PCR tubes or plate seal for 96 well plate
- Pipettes
- Aerosol-barrier pipette tips

C.4.2.4 Safety

- Lab coats
- Gloves
- Eye protection (safety glasses)

C.4.2.5 Procedure

1. Prepare a master mix for each reaction with a 10% surplus volume to allow for pipetting error according to the table below.

Reagents	Volume per single 25 µL reaction (µL)
2x SYBR® Green Quantitative RT-PCR buffer QR0100	12.5
Ref dye (optional) (instrument variable – 0.025-0.25 µL)	0.025
Primer F (10 µM)	1.125
Primer R (10 µM)	1.125
Total RNA RNA template – variable (250-2500 ng)	1
PCR grade water	9.1
MMLV RT enzyme	0.125

2. Combine reagents in 0.2 mL micro-tubes. If using a PCR plate, follow a plate schematic to ensure that the reaction mix, samples and controls are added to the correct wells.
3. After sealing each reaction, vortex gently to mix contents.
4. Centrifuge briefly to collect components at the bottom of the reaction tube.
5. Set the real time programme for:

Cycling conditions	Temp (°C)	Time
First Strand Synthesis	42-44	30 minutes
Denaturation/RT inactivation	94	30 seconds
Step 3 (40 cycles)	95	5 seconds
	55	15 seconds
	72	10 seconds

6. Perform post-reaction melt analysis.

C.4.3 Standard reverse transcription protocol (one-step probe detection)

C.4.3.1 Equipment

- Real-time PCR instrument

C.4.3.2 Reagents

- RNA (1 µg/µL)
- Quantitative RT-PCR Ready Mix (Sigma QR0200)
- Probe
- MMLV RT enzyme
- PCR grade water
- Primers

C.4.3.3 Supplies

- Laminar flow hood for PCR set up (optional)
- 1.5 mL tubes, sterile
- Tube racks for 1.5 mL tubes
- 0.2 mL PCR tubes
- Caps for PCR tubes
- Pipettes
- Aerosol-barrier pipette tips

C.4.3.4 Safety

- Lab coats
- Gloves
- Eye protection (safety glasses)

C.4.3.5 Procedure

1. Prepare a master mix for each reaction with 10% extra volume to allow for pipetting error (e.g. for 5 reactions prepare the equivalent volume of master mix required for 5.5 reactions).

Reagent	Volume per single 25 µL reaction (µL)
Quantitative RT-PCR 2x buffer QR0200	12.5
Ref dye (optional) (instrument variable – 0.025-0.25 µL)	0.025
Primer F (10 µM)	1.125
Primer R (10 µM)	1.125
Probe (10 µM)	0.625
Total RNA RNA template – variable (250-2500 ng)	1
PCR grade water	8.475
MMLV RT enzyme	0.125

2. Combine reagents in 0.2 mL micro-tubes.

3. After sealing each reaction, vortex gently to mix contents. Centrifuge briefly to collect components at the bottom of the reaction tube.

4. Set the real time programme for :

Cycling conditions	Temp (°C)	Time
First Strand Synthesis	42-44	30 minutes
Denaturation/RT inactivation	94	30 seconds
Step 3 (40 cycles)	94	5 seconds
	55	15 seconds
	72	10 seconds

C.5 Primer optimisation

Optimisation of primer concentration and annealing temperatures are an important step in the development of a robust assay. Signs of poor optimisation are lack of reproducibility between replicates and inefficient assays.

C.5.1 Concentration optimisation

C.5.1.1 Experimental objective:

When optimising primers, a matrix of reactions needs to be performed. This is used to test a range of primer concentrations against each other. An experiment testing six concentrations (e.g. 50 nmol to 800 nmol) requires a 6x6 matrix. The quantities stated in this protocol will allow each reaction to be performed in duplicate.

C.5.1.2 Equipment

- Quantitative PCR machine

C.5.1.3 Reagents

- cDNA diluted 1:10 or gDNA
- KiCqStart SYBR® Green ReadyMix™ (Sigma KCQS00/KCQS01/KCQS02/KCQS03 – depending on instrument)
- PCR grade water
- Forward and reverse primers for specific targets (stock at 100 µM)

C.5.1.4 Supplies

- Laminar flow hood for PCR set up (optional)
- 1.5 mL tubes, sterile
- Tube racks for 1.5 mL tubes
- 96 well plate
- Plate seal for 96 well plate
- Pipettes
- Aerosol-barrier pipette tips

C.5.1.5 Safety

- Lab coats
- Gloves
- Eye protection (safety glasses)

C.5.1.6 Procedure

Notes for this protocol:

- cDNA is generated using a random primer or oligo-dT priming method and diluted 1:10 for use;
- All samples are processed in duplicate according to the plate layout below:

		nM Forward Primer											
nM Reverse Primer		50		100		200		400		600		800	
	50												
	100												
	200												
	400												
	600												
	800												
	NTC												

C.5.1.6.1 Reaction

1. Prepare primer dilutions as follows starting from 100 µM stock solution:

Note: 2.0 µL of each primer will be added to the reaction of 20 µL total volume. For this reason, the working concentrations of the primer stocks are at 10 times the desired final concentration.

a) Prepare 10 µM stocks of each primer

b) Using the 10 µM stock, make a serial dilution of both primer stocks to 0.5, 1, 2, 4, 6 and 8 µM as shown in the table below.

Final concentration (µM)	Volume H_2O (µL)	Volume 10 µM Stock (µL)	Total Volume (µL)
0.5	47.5	2.5	50
1	45	5	50
2	40	10	50
4	30	20	50
6	20	30	50
8	10	40	50

2. Prepare a qPCR master mix according to table below. Mix well.

Reagents	Volume per single 20 µL reaction (µL)	Volume for whole plate (µL) (84 reactions + 10% contingency= 92.4 reactions)
2x KiCqStart SYBR® Green qPCR ReadyMix	10	924
PCR grade water	3	277.2
Reference dye (Optional)	1	92.4
cDNA (1:10 of stock)	2	*
Forward Primer	2	*
Reverse Primer	2	*

***Do not add cDNA and primers until step #5**

3. Remove 184.8 µL of master mix from step 2 into a separate tube to use for setting up the No Template Control (NTC).

4. Add 26.4 µL of PCR grade water to the NTC mix in #3.

Note: Set NTC mix on ice until later use

5. Add 158.4 µL of cDNA template to the remaining master mix from step #2 (72 plus 10% to allow for pipetting error). Place master mix on ice.

6. Aliquot 16 µL master mix from step #5 into the PCR plate.

7. Aliquot 16 µL master mix from step #4 into the PCR plate for NTC.

8. Add 2.0 µL of appropriate reverse primer dilutions into the PCR plate.

9. Add 2.0 µL of appropriate forward primer dilutions into the PCR plate.

10. Seal plates and label. **(Make sure labelling does not obscure instrument excitation/detection light path)**

11. Process samples using the protocol below.

Cycling conditions	Temp (ºC)	Time (s)
Step 1	95	30
Step 2 (40 Cycles)	95	5
	60	30

Note: Use standard dissociation curve protocol (data collection)

C.5.2 Primer optimisation using temperature gradient

C.5.2.1 Experimental objective

One approach to assay optimisation requires the determination of the optimum Ta (annealing temperature) by testing identical reactions, containing a fixed primer concentration, across a range of annealing temperatures. In these reactions, primers are incorporated at a final concentration of 450 nM.

C.5.2.2 Equipment

- Quantitative PCR machine

C.5.2.3 Reagents

- cDNA diluted 1:10 or gDNA
- KiCqStart SYBR® Green ReadyMix™ (Sigma KCQS00/KCQS01/KCQS02/KCQS03: instrument specific)
- PCR grade water (Sigma W1754)
- Forward and reverse primers for test gene (stock at 100 µM)

C.5.2.4 Supplies

- Laminar flow hood for PCR set up (optional)
- 1.5 mL tubes, sterile
- Tube racks for 1.5 mL tubes
- 12-well PCR strip tubes
- Caps for 12-well PCR strip tubes
- Pipettes
- Aerosol-barrier pipette tips

C.5.2.5 Safety

- Lab coats
- Gloves
- Eye protection (safety glasses)

C.5.2.6 Procedure

Notes for this protocol:

- cDNA is generated using random priming or oligo-dT priming method and diluted 1:10 for use;
- 10 µM working solution of primers are prepared from 100 µM stock solutions;
- All reactions are performed in duplicate as technical replicates according to the following layout:

Temperature gradient												
	54	TM	TM	TM	TM	TM	TM	TM	TM	TM	TM	70
	GO1	GO1	GO1	GO1	GO1	GO1	GO1	GO1	GO1	GO1	GO1	GO1
	GO1	GO1	GO1	GO1	GO1	GO1	GO1	GO1	GO1	GO1	GO1	GO1
	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC
	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC

This table shows the distribution of the samples and controls across the temperature gradient. If the instrument has a temperature gradient that varies vertically across the plate column rather than horizontally, the samples and controls will need to be re-arranged accordingly.

C.5.2.6.1 Reaction

1. Prepare a master mix for 56 reactions according to table below. Mix well, avoiding bubbles.

Reactions	Volume per single 20 µL reaction (µL)	Volume for 56 reactions (µL)
KiCqStart SYBR® Green qPCR ReadyMix 2X	10	560
Forward primer (10 µM working solution)	0.9	50.4
Reverse primer (10 µM working solution)	0.9	50.4
PCR grade water	4.2	235.2

2. Remove 448 µL of master mix from step #1 (i.e. half) into a separate tube for setting up the No Template Control (NTC) reactions.

3. Add 112 µL of cDNA template to the remaining master mix from step #2. Place the template master mix on ice.

4. Add 112 µL of water to the other half of the master mix from step #2. Place the NTC master mix on ice.

5. Aliquot 20 µL template master mix from step #3 into two rows of the PCR plate.

6. Aliquot 20 µL NTC master mix from step # into two rows of the PCR plate.

7. Cover plates and label. **(Make sure that the labelling does not obscure instrument excitation/detection light path.)**

8. Process samples according to the three-step protocol below (note these conditions are specific for FAST cycling protocols).

FAST Cycling Conditions	Temp (ºC)	Time (s)
Step 1	95	30
Step 2	95	5
40 cycles	54-70 (gradient)	15
	72	10

Note: Use standard dissociation curve protocol (data collection)

C.6 qPCR reference gene selection protocol

C.6.1 Experimental objective

Gene expression data require a stable reference or loading control. This is usually one or more reference genes. Suitable reference genes are those which are unaffected by differences in samples and experimental treatments, and these must be determined for each experimental model.

C.6.2 Equipment

- Quantitative PCR machine

C.6.3 Reagents

- cDNA diluted 1:100
- KiCqStart SYBR® Green ReadyMix™ (Sigma KCQS00/KCQS01/KCQS02/KCQS03 – depends on instrument)
- PCR grade water
- Forward and reverse primers for test reference gene (stock at 100 µM)

C.6.4 Supplies

- Laminar flow hood for PCR set up (optional)
- 1.5 mL tubes, sterile
- Tube racks for 1.5 mL tubes
- 0.2 mL PCR tubes or 96 well plate
- Caps for PCR tubes or plate seal for 96 well plate
- Pipettes
- Aerosol-barrier pipette tips

C.6.5 Safety

- Lab coats
- Gloves
- Eye protection (safety glasses)

C.6.6 Procedure

Notes for these protocols:

- cDNA is generated using e.g. random priming or oligo-dT method (section C.4);
- Sufficient primers from stocks at 100 µM are diluted to 10 µM;
- If using a PCR plate, follow a plate schematic to ensure that the reaction mix, samples and controls are added to the correct wells;
- Test a wide range of designs for genes that may be potential reference genes, e.g. some primer sequences for human targets would be:

Reference gene	Sequence
CANX Forward	AAGTGGTTGCTGTGTATGTT
CANX Reverse	TTGGGAGATGAAGGAGGAG
HPRT1 Forward	GCCTAAGATGAGAGTTCAAGTT
HPRT1 Reverse	AACAACAATCCGCCCAAA
PGK1 Forward	GAGATGATTATTGGTGGTGGAA
PGK1 Reverse	AGTCAACAGGCAAGGTAATC
TBP Forward	CTCCCGGAATCCCTATCTT
TBP Reverse	GCCTTTGTTGCTCTTCCA
EIF4A2 Forward	GATCATGTCTGGTGGCTC
EIF4A2 Reverse	TCAAAGTTATCAACAATCTCATTCC
YWHAZ Forward	AACTTTTGTCTGCCTCATTCT
YWHAZ Reverse	GTAACATAAACCTGTCATAAATCGTAA
ATP5B Forward	TGGTGACTGGTATCAAGGT
ATP5B Reverse	GCTTTGGCGACATTGTTG
SDHA Forward	TGCTAAAGTTTCAGATTCCATTTC
SDHA Reverse	CCAGTTGTCCTCCTCCAT
TUBB2B Forward	GCTGGAGAGAATCAATGTTTACTA
TUBB2B Reverse	ACGAAATTGTCTGGTCTGAAG
UBC Forward	TTTAGGACGGGACTTGGG
UBC Reverse	CACAGCGATCCACAAACA
PPIA Forward	GCTCGCAGTATCCTAGAATC
PPIA Reverse	CAACAAACATTGACACTTCCT
GUSB Forward	GACACGCTAGAGCATGAG
GUSB Reverse	TCAGTCAGGTATTGGATGGT
RPLPO Forward	ACAATGGCAGCATCTACA
RPLPO Reverse	GTAATCCGTCTCCACAGA
GAPDH Forward	GGTCGGAGTCAACGGATT
GAPDH Reverse	ATCGCCCCACTTGATTTTG
TUBA1A Forward	CTTCCACCCTGAGCAACT
TUBA1A Reverse	TCCAACACGAGGTCAATGA
ACTB Forward	GGCACCCAGCACAATGAAGA
ACTB Reverse	AGGATGGAGCCGCCGATC
GAPDH Forward	TATGACAACAGCCTCAAGAT
GAPDH Reverse	GAGTCCTTCCACGATACC
Alu Forward	TGGTGAAACCCCGTCTCTACTAA
Alu Reverse	CCTCAGCCTCCCGAGTAGCT

C.6.6.1 Reaction

1. Prepare one qPCR master mix for each primer pair. Prepare sufficient master mix for two reactions per sample (aim for five test samples and five control samples) and two No Template Controls (NTC), incorporating an additional 10% volume to allow for pipetting error. **DO NOT ADD cDNA** to the master mix. Mix well and avoid bubbles.

Reactions	Volume per single 20 µL reaction (µL)
2x KiCqStart SYBR® Green qPCR ReadyMix	10
Forward primer (10 µM)	0.9
Reverse primer (10 µM)	0.9
PCR grade water	3.2

2. Add 15 µL of master mix to the defined tubes/wells

3. Add 5 µL of appropriate template to the defined tubes/wells (sample or water for NTC)

4. Cap tubes or seal plates and label. **(Make sure that the labelling does not obscure instrument excitation/detection light path.)**

5. Perform reactions according to the three-step protocol below.

Note: Use standard dissociation curve protocol (data collection)

Cycling Conditions	Temp (ºC)	Time (s)
Step 1	95	30
Step 2 (40 cycles)	95	5
	58	10
	72	15

C.7 qPCR efficiency determination protocol

Determination of reaction efficiency using cDNA standard curves over 10-fold and 2-fold dilution series.

C.7.1 Experimental objectives

Once an assay has been optimised it is important to verify the efficiency. This information is important when reporting and comparing assays. In this test the assay efficiency is compared over a wide and narrow dynamic range of cDNA concentrations, (although in practice a single working range encompassing the expected concentration of target within the samples is often selected).

C.7.2 Equipment

- Quantitative PCR machine

C.7.3 Reagents

- cDNA undiluted
- KiCqStart SYBR® Green ReadyMix™ (Sigma KCQS00/KCQS01/KCQS02/KCQS03 – depends on instrument)
- PCR grade water
- Forward and reverse primers for test genes (stock at 100 µM)

C.7.4 Supplies

- Laminar flow hood for PCR set up (optional)
- 1.5 mL tubes, sterile
- Tube racks for 1.5 mL tubes
- 0.2 mL PCR tubes or 96 well plate
- Caps for PCR tubes or plate seal for 96 well plate
- Pipettes
- Aerosol-barrier pipette tips

C.7.5 Safety

- Lab coats
- Gloves
- Eye protection (safety glasses)

C.7.6 Procedure

Notes for this protocol:

- cDNA is generated using e.g. random priming or oligo-dT method (section C.4.1). Do not dilute the RT product (a one-step approach can be adopted by diluting RNA and following the one-step RT-qPCR approach in section C.4.2);
- Dilute the forward and reverse primers for the test gene to 10 µM;
- The serial dilutions of the samples will be processed as duplicate reactions, using the following layout:

Plate layout for cDNA dilution					
Plate column	1	2	3	4	Rest of the plate
A	1	1	1	1	
B	0.1	0.1	0.5	0.5	
C	0.01	0.01	0.25	0.25	
D	0.001	0.001	0.125	0.125	
E	0.0001	0.0001	0.0625	0.0625	
F	0.00001	0.00001	0.03125	0.03125	
G	0.000001	0.000001	0.015625	0.015625	
H	NTC	NTC	NTC	NTC	

C.7.6.1 Reaction

1. Prepare a qPCR master mix that is sufficient for 40 reactions for amplification of the cDNA.

Reactions	Volume per single 20 µL reaction (µL)	Volume for 40 reactions (µL)
2x KiCqStart SYBR® Green qPCR ReadyMix	10	400
Forward primer (10 µM)	0.9	36
Reverse primer (10 µM)	0.9	36
PCR grade water – variable	4.2	168

2. Dilute the cDNA reactions through a series of 1:10 and 1:2 (as shown below).
3. Add 4 µL of appropriate template dilution to the defined wells (see table).
4. Add 16 µL of master mix to each well (see table).
5. Cap tubes or seal the plate and label. (**Make sure that the labelling does not obscure instrument excitation/detection light path**).
6. Process the samples according to the two-step protocol below.

Cycling conditions		
	Temp (ºC)	Time (s)
Step 1	95	30
Step 2 (40 cycles)	95	5
	60	30

Note: Use standard dissociation curve protocol (data collection)

C.8 qPCR gene expression analysis protocols

C.8.1 qPCR gene expression analysis (SYBR® Green)

The quantity of a gene transcript is measured relative to one or more stable reference genes using SYBR® Green.

C.8.1.1 Experimental objective

After optimisation of the assays for both target and reference genes, the assays are used to measure the quantity of target. A ratio is determined between the quantity of gene of interest (GOI) and the reference gene as described in section A.4.5.1.1

C.8.1.2 Equipment

- Quantitative PCR machine

C.8.1.3 Reagents

- cDNA (or other standard curve material) undiluted for standard curve
- Sample cDNA diluted 1:10
- KiCqStart SYBR® Green ReadyMix™ (Sigma KCQS00/KCQS01/KCQS02/KCQS03: instrument specific)
- PCR grade water
- Forward and reverse primers for test and reference genes (stock 100 µM)

C.8.1.4 Supplies

- Laminar flow hood for PCR set up (optional)
- 1.5 mL tubes, sterile
- Tube racks for 1.5 mL tubes
- PCR strip tubes or 96 well plate
- Caps for PCR strip tubes or plate seal for 96 well plate
- Pipettes
- Aerosol-barrier pipette tips

C.8.1.5 Safety

- Lab coats
- Gloves
- Eye protection (safety glasses)

C.8.1.6 Procedure

Notes for the following protocol

- cDNA is generated using e.g. random priming or oligo-dT method (section C.4);
- Dilute forward and reverse primers stock solution (100 µM) for genes of interest to 10 µM;
- If using a PCR plate, follow a plate schematic to ensure that the reaction mix, samples and controls are added to the correct wells;
- All tests will be processed as duplicate reactions.

C.8.1.6.1 Reaction

1. Prepare a qPCR master mix for each primer pair to be evaluated. Prepare sufficient mix for the standard curve reactions (described as six dilutions below), NTCs and for each sample, all in duplicate, incorporating an additional 10% volume to allow for pipetting error.

   (e.g. if there are five test samples, prepare a mix for the samples (5x2 reactions), the standard curve (6x2), and No Template Control (NTC) (1x2) = 24 reactions. Therefore prepare a mix for 26.4 or 27 reactions per primer pair.)

Reactions	Volume per single 20 µL reaction (µL)
2x KiCqStart SYBR® Green qPCR ReadyMix	10
Forward primer (10 µM)	0.9
Reverse primer (10 µM)	0.9
PCR grade water	3.2

2. Prepare a 1:10 fold serial dilution of suitable standard curve template/cDNA so that there is 20 µL of each dilution (six dilutions in total).

3. Add 5 µL of appropriate template serial dilution (standard curve) and water (NTC) to the defined tubes or wells.

4. Add 15 µL of master mix to each tube or well.

5. Cap tubes or seal the PCR plate and label. **(Make sure that the labelling does not obscure the instrument excitation/detection light path.)**

6. Process samples according to the protocol below.

Cycling conditions	Temp (ºC)	Time (s)
Step 1	95	30
Step 2 (40 cycles)	95	5
	60	30

Note: Use standard dissociation curve protocol (data collection)

C.8.2 qPCR Gene expression analysis using probe detection

The quantity of a gene transcript is measured relative to one or more stable reference genes using probe detection (single reactions).

C.8.2.1 Experimental objective

After optimisation of the assays for both target and reference genes, these assays are used to derive the quantity of target. A ratio is determined between the quantity of gene of interest (GOI) and the reference gene as described in section A.4.5.1.1

In the protocol below the probe is included at 250 nM and the primers at 450 nM. However, it is advisable to optimise these conditions and adjust the reaction volumes accordingly. For a multiplex reaction, all primers and probes are added to the reaction mix. It may be necessary to use a more concentrated stock for oligonucleotides.

C.8.2.2 Equipment

- Quantitative PCR machine

C.8.2.3 Reagents

- cDNA undiluted for standard curve
- Sample cDNA diluted 1:10
- LuminoCt qPCR Readymix (L6669)
- PCR grade water
- Forward and reverse primers for test and reference genes (stock 100 µM)
- Probe for each target (stock 100 µM)

C.8.2.4 Supplies

- Laminar flow hood for PCR set up (optional)
- 1.5 mL tubes, sterile
- Tube racks for 1.5 mL tubes
- 0.2 mL PCR tubes or 96 well plate
- Caps for PCR tubes or plate seal for 96 well plate
- Pipettes
- Aerosol-barrier pipette tips

C.8.2.5 Safety

- Lab coats
- Gloves
- Eye protection (safety glasses)

C.8.2.6 Procedure

Notes for the following protocol:

- cDNA is generated using e.g. random priming or oligo-dT method (section C.4);
- Dilute forward and reverse primers stock solutions (100 µM) for genes of interest to 10 µM;
- Dilute probe stock solutions (100 µM) to 10 µM;

- If using a PCR plate, follow a plate schematic to ensure that the reaction mix, samples and controls are added to the right wells;
- All test samples will be processed as duplicate reactions.

C.8.2.6.1 Reaction

1. Prepare qPCR master mixes for each primer pair to be analysed. Prepare sufficient mix for the standard curve reactions (described as six dilutions below), NTCs and for each sample, all in duplicate plus an extra 10% to allow for pipetting errors.

Reactions	Volume per single reaction 25 µl
2x LuminoCt qPCR ReadyMix	12.5
Forward primer (10 µM)	1.125
Reverse primer (10 µM)	1.125
Probe (10 µM)	0.625
PCR grade water	4.625

2. Prepare a 10-fold dilution of suitable standard curve template/cDNA, resulting in at least a volume of 20 µL at each dilution (six dilutions in total).
3. Add 5 µL of appropriate template serial dilution (standard curve) and water (NTC) to the defined tubes or wells.
4. Add 20 µL of master mix to each tube/well.
5. Cap tubes or seal the PCR plate and label. **(Make sure that the labelling does not obscure the instrument excitation/detection light path.)**
6. Process the samples according to the two-step protocol below.

Cycling conditions	Temp (ºC)	Time (s)
Step 1	95	30
Step 2 (40 cycles)	95	5
	60	30

Section D: Further resources

Recorded seminars

Sigma offers recorded seminars on the topics of qPCR and MIQE guidelines which are available from the following link:

http://www.sigmaaldrich.com/life-science/learning-center/customer-education/qpcr-miqe-seminar-series.html

qPCR topics

- Primer and Probe Design
- An Introduction to qPCR Concepts
- Selecting a qPCR Basic Detection Chemistry
- Choosing a Fluorophore/Quencher Combination
- Chemistries for More Challenging qPCR Assays
- MIQE Concepts
- Reference Gene Validation
- Data Analysis Guidelines

MIQE guidelines:

- MIQE: Assay Design Considerations
- MIQE: Sample Derived Inhibitors
- MIQE: RNA Quality Considerations
- MIQE: RNA Quantity and RT Considerations

OligoArchitect (assay design)

OligoArchitect is a primer and probe design tool supplied by Sigma and it includes functionality to help design across exon splice sites and achieve MIQE compliance.

http://www.sigmaaldrich.com/life-science/custom-oligos/dna-probes/product-lines/probe-design-services.html

Websites

Information on MIQE guidelines: www.miqe.info/ . Website curated by Stephen Bustin. [1]

www.gene-quantification.info/ Website edited by Michael Pfaffl.[2]

Useful links and downloads from Tataa Biocenter http://tataa.gene-quantification.info/

AutoPrime http://www.autoprime.de/AutoPrimeWeb

qPCR technology hub: http://www.technologynetworks.com/qPCR/

Genome web: http://www.genomeweb.com/tech-guide-archive

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Section E: Further reading

A to Z Quantitative PCR, IUL Press. Edited by Stephen Bustin (2004)

PCR technologies: Current innovations, 2nd edition. CRC Press Edited by Thomas Weissensteiner, Tania Nolan, Stephen A. Bustin and Hugh G. Griffin. (2003)

S. A. Bustin, T. Nolan, Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction, *J. Biomol. Tech.*, 15 (2004) 155-166

Vladimir Benes, Mirco Castoldi, Expression profiling of microRNA using real-time quantitative PCR, how to use it and what is available. *Methods*, 50 (2010) 244-249

Essentials of Nucleic Acid Analysis, RSC Publishing, Edited by Jacquie T Keer and Lyndsey Birch (2008)

Real-time PCR: Advanced Technologies and Applications, 3rd edition, Caister Academic Press, Saunders & Lee, 2013

Section F: References

1. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr; 55(4):611-22. Epub 2009 Feb 26. PubMed PMID: 19246619.

2. Rasmunsen R. Quantification on the light cycler. Wittwer CT, Meuer S, Nakagawara K. (ed) Rapid Cycle Real-time PCR, Methods and Applications. Springer Verlag, Heidelberg. 2001; page 21-34.

3. Wahlberg K, Huggett J, Sanders R, Whale AS, Bushell C, Elaswarapu R, Scott DJ, Foy CA. Quality Assessment of Biobanked Nucleic Acid Extracts for Downstream Molecular Analysis. Biopreservation and Biobanking. 2012;10(3): 266-275. doi:10.1089/bio.2012.0004.

4. Vermeulen J, De Preter K, Lefever S, Nuytens J, De Vloed F, Derveaux S, Hellemans J, Speleman F, Vandesompele J. Measurable impact of RNA quality on gene expression results from quantitative PCR. Nucleic Acids Res. 2011 May;39(9):e63. Epub 2011 Feb 11. PubMed PMID: 21317187; PubMed Central PMCID: PMC3089491.

5. Wilfinger WW, Mackey K, Chomczynski P. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. Biotechniques. 1997 Mar;22(3):474-6, 478-81.

6. Huberman JA. Importance of measuring nucleic acid absorbance at 240 nm as well as at 260 and 280 nm. Biotechniques 1995; 18:636.

7. Manchester KL. Value of A260/A280 ratios for measurement of purity of nucleic acids. Biotechniques 1995; 19:208–210.

8. Manchester KL. Use of UV methods for measurement of protein and nucleic acid concentrations. Biotechniques. 1996;20(6):968-70.

9. Huggett JF, Novak T, Garson JA, Green C, Morris-Jones SD, Miller RF, Zumla A. Differential susceptibility of PCR reactions to inhibitors: an important and unrecognised phenomenon. BMC Res Notes. 2008 Aug 28;1:70. PubMed PMID: 18755023; PubMed Central PMCID: PMC2564953.

10. Witt N, Rodger G, Vandesompele Jo, Benes V, Zumla A, Rook GA, Huggett JF. An assessment of air as a source of DNA contamination encountered when performing PCR. J Biomol Tech. 2009 December;20(5): 236–240. PMCID: PMC2777341.

11. Nolan T, Hands RE, Ogunkolade W, Bustin SA. SPUD: a quantitative PCR assay for the detection of inhibitors in nucleic acid preparations. Anal Biochem. 2006 Apr 15;351(2):308-10. Epub 2006 Feb 20. PubMed PMID: 16524557.

12. Newbury SF, Smith NH, Higgins CF. Differential mRNA stability controls relative gene expression within a polycistronic operon. Cell. 1987;51(6):1131-1143.

13. Personal communication: Tania Nolan, Sigma-Aldrich.

14. Stöcher M, Leb V, Hölzl G, Berg J. A simple approach to the generation of heterologous competitive internal controls for real-time PCR assays on the LightCycler. J Clin Virol. 2002; 25 Suppl 3(1):S47-S53, PubMed PMID: 12467777

15. Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nat Protoc. 2006;1(3):1559-82. PubMed PMID: 17406449.

16. Ståhlberg A, Håkansson J, Xian X, Semb H, Kubista M. Properties of the reverse transcription reaction in mRNA quantification. Clin Chem. 2004 Mar;50(3):509-15. Epub 2004 Jan 15. PubMed PMID: 14726469.

17. Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N. The real-time polymerase chain reaction. Mol Aspects Med. 2006 Apr-Jun;27(2-3):95-125. Epub 2006 Feb 3.Review. PubMed PMID: 16460794.

18. Udvardi M, Czechowski T, Scheible, WR. Eleven golden rules of quantitative RT-PCR. Plant Cell. 2008;20: 1736–1737.

19. Burrell A, Foy C, Burns M. Applicability of three alternative instruments for food authenticity analysis – GMO identification. Biotechnology Research International.2011;Article ID 838232,doi:10.4061/2011/838232. http://www.sage-hindawi.com/journals/btri/2011/838232/.

20. French DJ, Archard CL, Brown T, McDowell DG, HyBeacon probes: a new tool for DNA sequence detection and allele discrimination. Mol Cell Probes. 2001 Dec;15(6):363-74. PubMed PMID: 11851380.

21. Ramakers C, Ruijter JM, Deprez RH, Moorman AF. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett. 2003 Mar 13;339(1):62-6. PubMed PMID: 12618301.

22. Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, Moorman AF. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res. 2009 Apr;37(6):e45. Epub 2009 Feb22. PubMed PMID: 19237396; PubMed Central PMCID:MC2665230.7.

23. Ruijter JM, Pfaffl MW, Zhao S, Spiess AN, Boggy G, Blom J, Rutledge RG, Sisti D, Lievens A, De Preter K, Derveaux S, Hellemans J, Vandesompele J, (2013) Evaluation of qPCR curve analysis methods for reliable biomarker discovery: bias, resolution, precision, and implications Methods (59)1, 3-9, special issue “Transcriptional Biomarkers”, edited by Michael W. Pfaffl.

24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-8. PubMed PMID: 11846609.

25. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001 29 (9): e45.

26. Barwick V and Hopkins SL, An introduction to method validation. From Essentials of nucleic acid analysis, RSC Publishing, Edited by Jacquie T Keer and Lyndsey Birch (2008)

27. Youden WJ and Steiner EH, Statistical Manual of the AOAC, AOAC, 1975, ISBN 0-935584-15-3

28. Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation;strategies and considerations. Genes Immun. 2005 Jun;6(4):279-84. Review. PubMed PMID: 15815687.

29. Whale AS, Huggett JF, Cowen S, Speirs V, Shaw J, Ellison S, Foy CA, Scott DJ. Comparison of microfluidic digital PCR and conventional quantitative PCR for measuring copy number variation. Nucleic Acids Res. 2012 Jun;40(11):e82. Epub 2012 Feb 28. PubMed PMID: 22373922; PubMed Central PMCID: PMC3367212.

30. Dheda K, Huggett JF, Chang JS, Kim LU, Bustin SA, Johnson MA, Rook GA, Zumla A. The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. Anal Biochem. 2005 Sep 1;344(1):141-3. PubMed PMID: 16054107.

31. Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques. 2004 Jul;37(1):112-4, 116, 118-9. PubMed PMID: 15283208.

32. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A,Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002 Jun 18;3(7):RESEARCH0034. Epub 2002 Jun 18. PubMed PMID: 12184808; PubMed Central PMCID: PMC126239.

33. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnol Lett. 2004 Mar;26(6):509-15. PubMed PMID: 15127793.

34. Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, Vandesompele J. A novel and universal method for microRNA RT-qPCR data normalization. Genome Biol. 2009;10(6):R64. doi: 10.1186/gb-2009-10-6-r64. Epub 2009 Jun 16. PubMed PMID: 19531210; PubMed Central PMCID: PMC2718498.

35. D'haene B, Mestdagh P, Hellemans J, Vandesompele J. miRNA expression profiling: from reference genes to global mean normalization. Methods Mol Biol. 2012;822:261-72. doi: 10.1007/978-1-61779-427-8_18. PubMed PMID: 22144205.

36. Vermeulen J, De Preter K, Lefever S, Nuytens J, De Vloed F, Derveaux S, Hellemans J, Speleman F, Vandesompele J. Measurable impact of RNA quality on gene expression results from quantitative PCR. Nucleic Acids Res. 2011 May;39(9):e63. Epub 2011 Feb 11. PubMed PMID: 21317187; PubMed Central PMCID: PMC3089491.

37. Guescini M, Sisti D, Rocchi MB, Stocchi L, Stocchi V. A new real-time PCR method to overcome significant quantitative inaccuracy due to slight amplification inhibition. BMC Bioinformatics. 2008 Jul 30;9:326. PubMed PMID: 18667053; PubMed Central PMCID: PMC2533027.

38. Rutledge RG, Stewart D. Critical evaluation of methods used to determine amplification efficiency refutes the exponential character of real-time PCR. BMC Mol Biol. 2008 Oct 30;9:96. PubMed PMID: 18973660; PubMed Central PMCID: PMC2587475.

39. Narum SR. Beyond Bonferroni: Less conservative analyses for conservation genetics. Conservation Genetics. 2006; 7:783–787 DOI 10.1007/s10592-005-9056-y.

40. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 2002 May 1;30(9):e36. PubMed PMID: 11972351; PubMed Central PMCID: PMC113859.

41. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 2004 Aug 1;64(15):5245-50. PubMed PMID: 15289330.

42. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 2007;8(2):R19. PubMed PMID: 17291332; PubMed Central PMCID: PMC1852402.

43. Querci M, Foti N, Bogni A, Kluga L, Broll H, and Van den Eede G. Real-Time PCR-based ready-to-use multi-target analytical system for GMO detection. Food Anal Methods. 2009;2:325–336 DOI 10.1007/s12161-009-9093-0.

44. GMO multiplex screen protocol: Standard operating procedure for the simultaneous detection of general DNA targets associated with the presence of genetically modified soya and maize". Foodbase: G03022.

45. Dooley JJ, Paine KE, Garrett SD, Brown HM. Detection of meat species usingTaqMan real-time PCR assays. Meat Sci. 2004 Nov;68(3):431-8. PubMed PMID:22062411.

46. Bryan GJ, Dixon A, Gales ND and Wiseman G. A PCR-based method for the detection of hexaploid bread wheat adulteration of durum wheat and pasta, J Cereal Sci. 1998 28. 135-145.

47. Fryer JF, Baylis SA, Gottlieb AL, Ferguson M, Vincini GA, Bevan VM, Carman WF, Minor PD. Development of working reference materials for clinical virology. J Clin Virol. 2008 Dec;43(4):367-71. Epub 2008 Sep 26. Review. PubMed PMID:18823817.

48. Honeyborne I, McHugh TD, Phillips PP, Bannoo S, Bateson A, Carroll N, Perrin FM, Ronacher K, Wright L, van Helden PD, Walzl G, Gillespie SH. Molecular bacterial load assay, a culture-free biomarker for rapid and accurate quantification of sputum Mycobacterium tuberculosis bacillary load during treatment. J Clin Microbiol. 2011 Nov;49(11):3905-11. Epub 2011 Sep 7. PubMed PMID: 21900522; PubMed Central PMCID: PMC3209113.

49. Boehme CC, Nicol MP, Nabeta P, Michael JS, Gotuzzo E, Tahirli R,Tarcela Gler M, Blakemore R, Worodria W, Gray C, Huang L, Caceres T, Mehdiyev R, Raymond L, Whitelaw A, Sagadevan K, Alexander H, Albert H, Cobelens F,Cox H, Alland D, Perkins MD. Feasibility, diagnostic accuracy, and effectiveness of decentralised use of the Xpert MTB/RIF test for diagnosis of tuberculosis and multidrug resistance: a multicentre implementation study. Lancet. 2011 April 30;377(9776): 1495–1505. doi: 10.1016/S0140-6736(11)60438-8 PMCID: PMC3085933.

50. Rachow A, Zumla A, Heinrich N, Rojas-Ponce G, Mtafya B, Reither K, Ntinginya EN, O'Grady J, Huggett J, Dheda K, Boehme C, Perkins M, Saathoff E, Hoelscher M. Rapid and Accurate Detection of mycobacterium tuberculosis in sputum samples by Cepheid Xpert MTB/RIF assay—A clinical validation study. PLoS One. 2011;6(6): e20458. Published online 2011 June 29. doi: 10.1371/journal.pone.0020458 PMCID: PMC3126807.

51. Ambros V. The functions of animal microRNAs. Nature. 2004 Sep 16;431(7006):350-5. Review. PubMed PMID: 15372042.

52. Kloosterman WP, Plasterk RH. The diverse functions of microRNAs in animal development and disease. Dev Cell. 2006 Oct;11(4):441-50. Review. PubMed PMID: 17011485.

53. Rosenfeld N, Aharonov R, Meiri E, Rosenwald S, Spector Y, Zepeniuk M, Benjamin H, Shabes N, Tabak S, Levy A, Lebanony D, Goren Y, Silberschein E, Targan N, Ben-Ari A, Gilad S, Sion-Vardy N, Tobar A, Feinmesser M, Kharenko O, Nativ O, Nass D, Perelman M, Yosepovich A, Shalmon B, Polak-Charcon S, Fridman E, Avniel A, Bentwich I, Bentwich Z, Cohen D, Chajut A, Barshack I. MicroRNAs accurately identify cancer tissue origin. Nat Biotechnol. 2008;26(4):462-9.

54. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen KS, Stirewalt DK, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008 July 29;105(30): 10513–10518. Published online 2008 July 28. doi: 10.1073/pnas.0804549105 PMCID: PMC2492472.

55. Jepsen JS, Sørensen MD, Wengel J. Locked nucleic acid: a potent nucleic acid analog in therapeutics and biotechnology. Oligonucleotides. 2004;14(2):130-46.

56. Balcells I, Cirera S, Busk PK. Specific and sensitive quantitative RT-PCR of miRNAs with DNA primers. BMC Biotechnol. 2011;11:70.

57. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Anderson MR, Lao KQ, Livak KJ, and Guegle KJ. Real-time quantification of micrRNAs by stem-loop RT-PCR. NAR, 2010, 33(20):e179.

58. Benes V and Castoldi M. Expression profiling of microRNA using real-time quantitative PCR, how to use it and what is available. Methods, 2010, 50:244-249.

59. Castoldi M, Spasia MV, Altamura S, Elman J, Lindow M, Kiss J, Stolte J, Sparla R, D'Alessandro LA, Klingmullers U, Fleming RE, Longerich T, Grones HJ, Benes V, Kauppinen S, Hentze MW and Muckenthaler MU. The liver-specific microRNA miR-122 controls systematic iron homeostasis in mice. JIC, 2011, 121(4):1386-1396.

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