qPCR Technical Guide

PCRBIOSYSTEMS

simplifying research

1. Introduction to qPCR

1.1 What qPCR is & how it works

Quantitative PCR (qPCR) is one of the foremost methods for detection and quantification of nucleic acid targets. The technique is also known as real-time qPCR and is not to be confused with RT-qPCR (Reverse Transcription-qPCR). It relies on the same basic reagents, enzyme and thermocycling conditions as classic PCR but with additional reagents that generate fluorescence in proportion to the amount of product present in a reaction. Specialised thermocyclers, equipped with fluorometer, are then used to record the signal in real time (Figure 1). This fluorescence is measured in arbitrary or relative fluorescence units (AFU and RFU, respectively).

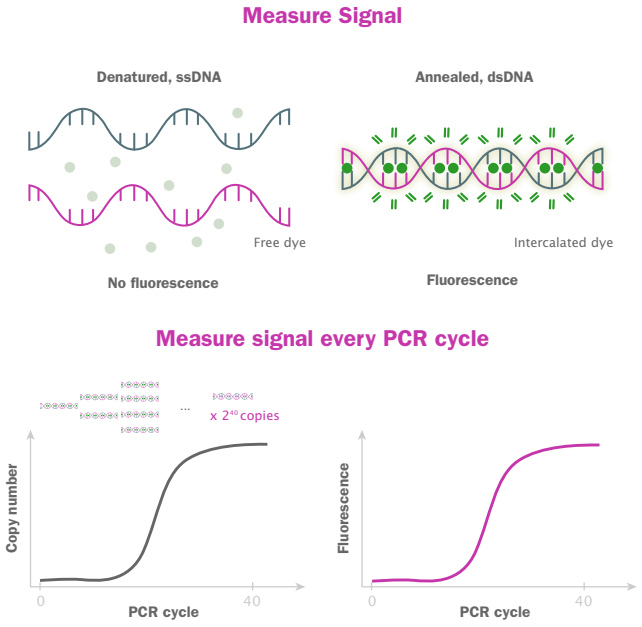


Figure 1

Dye-based fluorescent signal generation in qPCR

Traditional endpoint PCR only allows semi-quantitative evaluation of initial target abundance. This is both because PCR efficiency is not maintained in later cycles, due to product inhibition of DNA polymerase, and depletion of reagents. Conversely, because qPCR enables real-time monitoring of product accumulation, it can yield more accurate quantitative information. This is extracted from the amplification curve generated by plotting qPCR reaction fluorescence intensity against PCR cycles.

A typical qPCR amplification curve is sigmoidal. Initially, fluorescence increases in a linear-ground, or initiation phase, below background noise. As the product accumulates exponentially, fluorescence increases proportionally and passes the background noise level. Fluorescence continues to increase every cycle in a log-linear manner until PCR reagents are depleted. Subsequently, fluorescence accumulation reaches a plateau phase until the end of the cycling program, due to the reaction slowing down or stopping (Figure 2). Aberrations in this shape of the amplification plot are indicative of problems with the reaction. To understand more about how to interpret such aberrations and what steps are needed to rectify them, see the “Troubleshooting ” section on page 34.

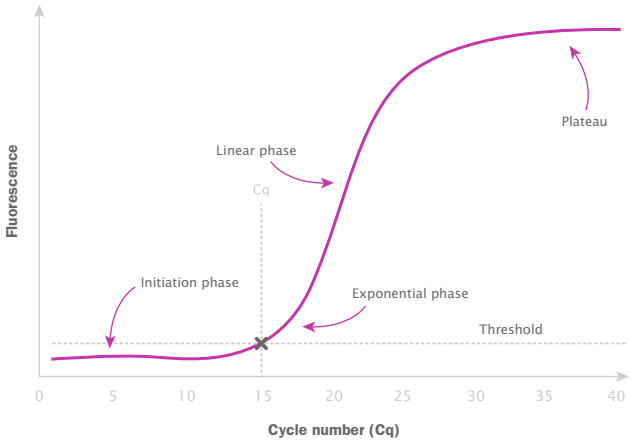


Figure 2

Generic qPCR amplification plot

The most valuable metric derived from the qPCR amplification curve is the so-called threshold cycle (Ct) or quantification cycle (Cq). This corresponds to the PCR cycle at which the fluorescent signal generated by the target product crosses an arbitrary threshold level above background noise (Figure 2). This Cq value is proportional to the initial target concentration and can be used to compare the amount of target among different samples . Alternatively, it can be compared to a standard curve of reference sample Cqs with known target quantities to enable initial template quantification.

2. Detection methods in qPCR

Two different chemical strategies can be used to enable real-time monitoring of product accumulation via fluorescence: dye-based detection and probe-based detection.

2.1 Dye-based detection

This approach to real time detection of PCR products is achieved by adding a fluorescent dye capable of intercalating in double stranded DNA molecules. The dye itself emits a baseline of fluorescence when free in solution, but the intensity of fluorescence increases dramatically when the same number of dye molecules stack up between DNA base pairs. Thus, at the start of a qPCR reaction, a basal level of fluorescence is emitted by the free dye, in addition to the small amounts of dye intercalated in double stranded DNA present at the beginning of the reaction (which should be present in low amount).

As the PCR progresses, the amount of double stranded DNA (dsDNA) in the sample increases. Correspondingly, the ratio of intercalated over free dye increases and thus the fluorescence intensity increases proportionally (Figure 3). After several cycles, fluorescence reaches a plateau, due to the PCR reaction slowing down to a halt, leading to the sigmoidal data curve of emitted fluorescence discussed previously. In many cases, particularly with older qPCR instruments, an additional passive dye (usually ROX) is included in the qPCR mix to normalise fluorescence signal variations across a run.

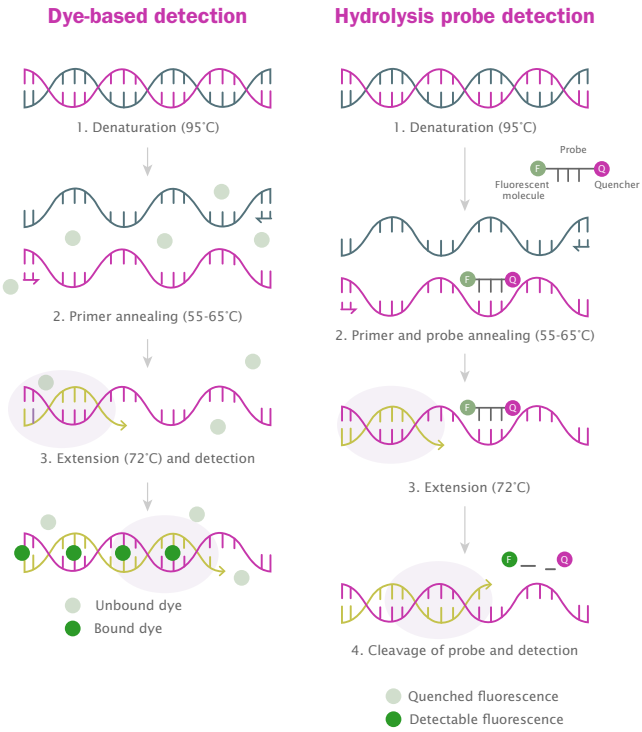


Figure 3

Dye versus hydrolysis probe signal generation mechanisms in qPCR

Benefits and shortcomings of dye-based qPCR

Benefits

1. The dye can be used to detect any target molecule of choice when combined with a specifically designed primer pair. This means that high throughput independent qPCRs targeting multiple different targets can be designed in a simple and cheap manner.
2. The use of a DNA intercalating dye leads to the reversible accumulation of fluorescent signal. Since strong fluorescence is only emitted by intercalated dye, denaturation of DNA leads to a reduction in fluorescent signal. This enables the testing of a reaction's specificity using melt curve analysis.

Shortcomings

1. Non-specific fluorescent signal will be generated by any dsDNA which binds the intercalating dye. Thus, any spurious products derived from mis-priming, the presence of similar sequences to the target in the sample, the formation of primer dimers and potential primer hairpin structures can all lead to the accumulation of spurious signal during the reactions. To validate results, melt curve analysis, gel electrophoresis of the endpoint product, or Sanger sequencing should be performed.
2. Only one target can be assayed in any reaction, thus multiplex reactions aren't feasible. If multiple targets are to be quantified in a sample, a corresponding number of reactions must be set up.

2.2 Melt curve analysis

A melt curve analysis allows the identification of a qPCR product's melting point. This information is useful as a proxy for establishing qPCR product specificity. For a melt curve analysis to be carried out, an additional thermocycling step is added at the end of the qPCR cycling program. During this step, the reaction samples are slowly denatured by a gradual increase of the incubation temperature (usually from 50-55 ˚C to 95-99 ˚C) while fluorescence is collected in a stepwise manner every 0.1-0.5 ˚C. The accumulated data points of fluorescence intensity plotted against the temperature generate the melt curve.

In a reaction containing one specific product, fluorescence will remain steady until the temperature approaches the target's melting point and fluorescence then decreases rapidly to baseline. The point at which fluorescence drops to 50% of maximum is considered that molecule's Tm (Figure 5).

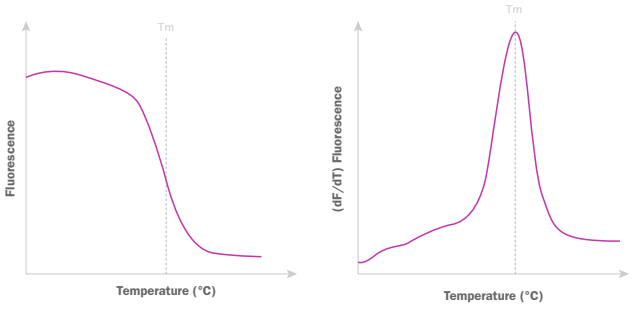


Figure 5

Example qPCR melt curve and resulting melt peak

While the melt curve itself is informative, an easier way to evaluate reaction specificity is to plot the first-order derivative of the acquired fluorescence intensity (-dRFU) over the change in temperature (dT), i.e., dRFU/dT. The resulting diagram for a qPCR reaction containing one specific product is a parabolic peak centred around the product's Tm and is called a melt peak (Figure 5).

Leading, skewed or multiple peaks are all strong indications of non-specific products accumulating in the reaction and require further investigation prior to conducting analysis for template quantification (Figure 6).

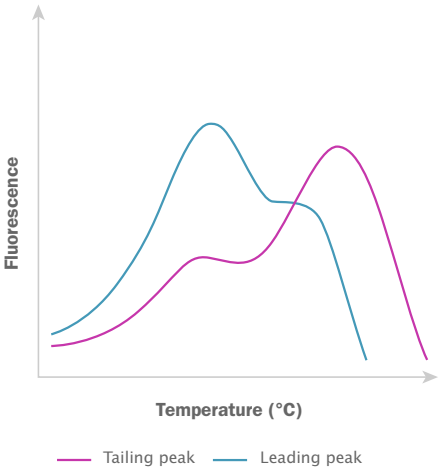


Figure 6

Examples of problematic melt peaks, tailing and leading peaks indicate primer dimers

When conducting a melt curve analysis, it is important to validate all primer pairs the first time they are used, by running the final PCR product on an agarose gel to ensure that an observed melt peak corresponds to the expected target product size. While the melt curve/peak is a strong indicator of specificity, it is important to note that some targets with a similar length and sequence can have the same melt curves. Additionally, different reagents, varying ionic strength, and random contaminants present in different samples may cause a shift in the observed melt peak, even when the desired target has been amplified with high specificity (Figure 7).

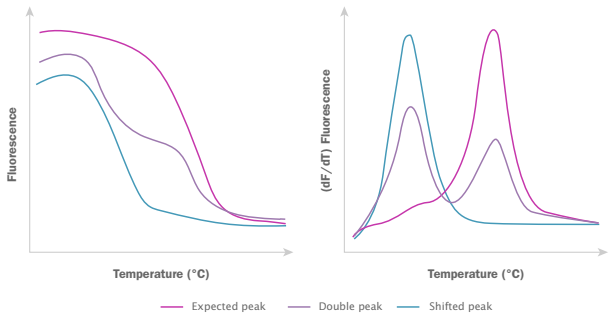


Figure 7

Examples of problematic melt peaks, shifted or double peaks indicate non-specific product amplification

Abnormal melt peaks will also be observed when multiple products accumulate in a reaction, such as multiple products formed by off-target priming, and primer dimer or primer hairpin formation. Multiple products in a reaction will generate a corresponding number of, usually distinct, melt peaks each centred on a different temperature (Figure 7). In some instances, multiple products will appear as a double or a leading peak. Primer dimers or hairpins will usually appear at lower temperatures than full length products, although this is not always the case.

Sometimes certain amplicons produce a double peak even when there is no other product present. This can happen when the amplicon sequence has a great difference in GC-content at distinct regions. Such an uneven base pair content leads to partial denaturation of low GC regions first, followed by denaturation of the GC-rich regions at a higher temperature, ultimately giving the appearance of two distinct peaks. A notable example is the mammalian CFTR gene2. While this phenomenon is rare, it is something to be aware of when carrying out qPCR analyses.

2.3 Probe-based detection

There are three different types of qPCR probes based on differing mechanisms of action: hydrolysis, dual hybridization, and hairpin probes. Fluorescence detection with all of these strategies rely on a physical phenomenon known as FRET. We will focus primarily on hydrolysis probes (otherwise known as TaqMan® probes) but will briefly describe the other types for completeness.

Förster Resonance Energy Transfer (FRET)

FRET describes a physical phenomenon in which two light-sensitive molecules transfer energy in a non-fluorescence dependant manner. During this phenomenon one of the two molecules acts as a donor and in its electronically excited state can transfer energy to the other, acceptor, molecule, exciting this in turn. Depending on the nature of the acceptor molecule, it may release this energy in the form of fluorescence or may dissipate energy in a non-light dependent manner. In this latter case, the acceptor molecule acts as a quencher. Simultaneously, the donor molecule drops to its non-excited electronic state without emitting fluorescence.

Detection of the target product using **hydrolysis probes** is achieved through the addition of an oligonucleotide-probe designed to be complementary to a section of the target molecule. The probe is covalently modified to include a fluorophore at one end and a quencher molecule, which absorbs (quenches) light emitted from the fluorophore only in its proximity, at the other end of the sequence. Sometimes a second quencher (added in the middle of the probe sequence) may be required to lower background signal. The probe is also designed to have an annealing temperature similar to that of the primer pair used to amplify the target.

When included in the qPCR reaction mix initially, no fluorescent signal is produced as the quencher(s) absorb(s) the signal. As the thermocycling program begins, the probe anneals with the target during the denaturation/annealing phase of the program. During extension, the polymerase generating the new strand of DNA degrades the annealed probe through its 5'-3' exonuclease activity, freeing the quencher and fluorophore molecules in the reaction mix. Because the distance between these two molecules is now much greater, the fluorophore, once stimulated, can emit a detectable fluorescent signal (Figure 3).

As each cycle is repeated, increasing amounts of target DNA molecules lead to an increasing amount of probe being degraded and to more and more fluorescent signal emitted from the released fluorophore molecule. This generates a series of data points from each cycle to give the expected sigmoidal curve from which the Cq cycle value? is calculated. As with dye-based detection, a passive reference dye can be included to normalise fluorescent signal variation.

Note:

A point to note is that not all qPCR mixes contain polymerases with 5'-3' exonuclease activity. As such, an appropriate probe-detection mix should be chosen when this type of detection is desired.

Hairpin probe (molecular beacons) detection utilises one probe that is covalently modified to carry a quencher and fluorophore molecule, like the hydrolysis probes. However, the sequence is also designed to form a hairpin structure, which brings its covalently modified bases into proximity, thereby ensuring strong fluorophore quenching when the probe forms the hairpin and is unbound to the target. Numerous variations of this type of probe exist with varying relative positions of the quencher and fluorophore and depending on whether the hairpin is resolved during the denaturation step or the annealing step thereby altering when signal generation occurs[1].

Dual hybridization probes rely on the presence of two probes that are specific to the qPCR target. Both are designed to bind consecutively to adjacent segments on the same strand of the target, with the first probe covalently modified to carry a FRET donor molecule at the 3' end and the second probe covalently modified to carry a FRET acceptor molecule at the 5' end. When both probes bind the target and the donor molecule is stimulated by an appropriate excitation wavelength, there is a transfer of excitation energy to the acceptor molecule via FRET, which in turn emits fluorescence at its corresponding wavelength recorded by the qPCR instrument.

The acceptor and donor molecules can be designed the other way around on the two primers, provided they are adjacent to each other when both probes are bound to the target. For FRET to be efficient enough for the dual probe system to work, the acceptor and donor molecules must be within a 10 Å radius, thus the probes should bind adjacently to each other on the target molecule (< 3 bp apart).

Advantages to probe-based detection:

1. Higher specificity, compared to dye-based detection.
2. The ability to multiplex primer sets and probes.

Higher specificity is achieved because fluorescence is only generated after the probe has annealed to the target molecule, ensuring that no signal is generated by non-specific products. This is different to the intercalating dyes, which generate signal regardless of the double stranded DNA sequence they bind too.

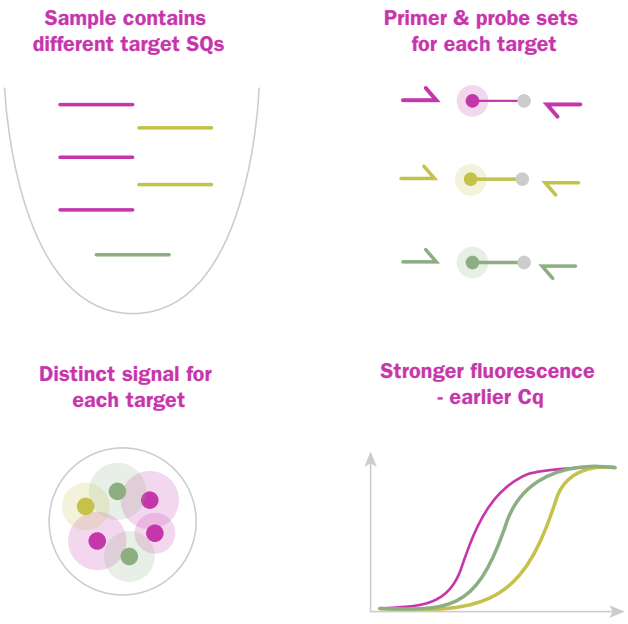


Figure 4

Multiplex qPCR signal generation via probes with distinct fluorophores

Due to this specificity and the possibility to use different fluorophores, this system can be used to set up multiplexed reactions in which single qPCR reaction mixes are used to detect multiple targets simultaneously. This is achieved simply by adding specific oligonucleotide probes labelled with spectrally distinct fluorophores, each of which can yield specific quantitative information on separate targets in one sample. Multiplexing is an extremely useful approach allowing the efficient, accurate quantification of multiple targets in a sample both in research and diagnostic settings (Figure 4).

Drawbacks of probe-based qPCR

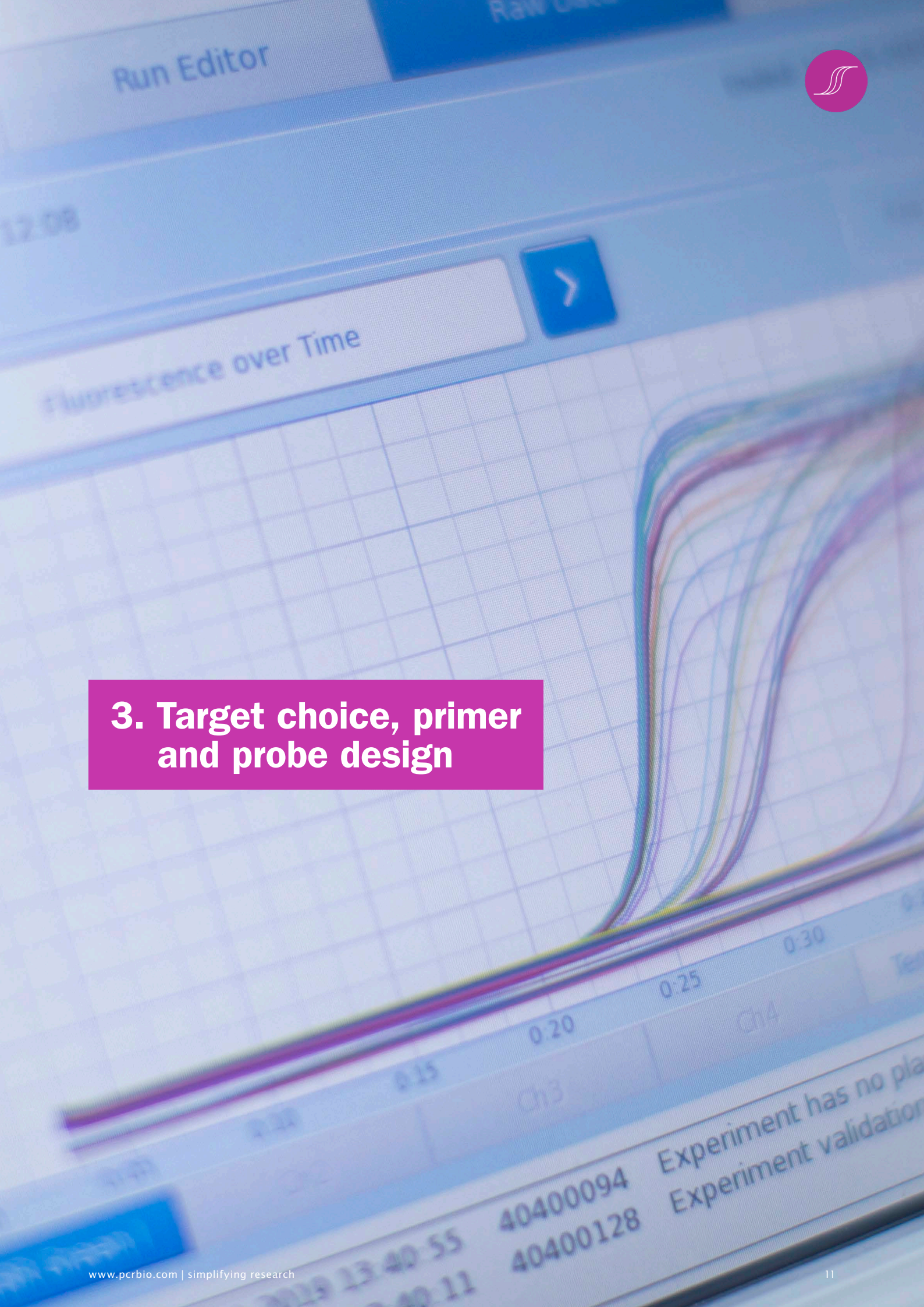
1. The increased time and attention needed to design specific probes for quantification.
2. Added cost of purchasing covalently modified oligonucleotide probes in order to include the fluorophore and quencher molecules.
3. The need to design and validate a set of primers and probe for every new target investigated.

Primer dimers, hairpins, or probe secondary structures, affect also probe-based qPCR, because the formation of such structures reduces the amount of primers and probe available to bind the real target. This competition usually results in delayed Cqs.

Both dye and probe-based detection methods offer specific advantages and disadvantages. Critically, both methods can be affected by PCR artefacts arising from excessive cycling, although dye-based qPCR is more susceptible to generating erroneous data because of such artefacts. The “Troubleshooting” section addresses such issues.

Table 1: Pros and cons to dye and probe-based qPCR

Consideration	Dye-based detection	Probe-based detection
Cost	Low cost: enzyme mix and one pair of primers per target	Higher cost: probe mix, one primer pair, at least one high purity oligo probe (modified oligo) per target
Experimental design	Easy: select sequence-specific primers for each target, ensure similar annealing temperature.	More complex: Design primers and probes with suitable annealing temperature and high specificity, each new target requires an additional probe.
Specificity	Potential for non-specific signal from any dsDNA, including primer dimers and non-specific products	High specificity, only products with high sequence identity to the probe sequence generate signal. Potential Ct (Cq) delay due to primer dimers
Multiplexing compatibility	No	Yes
Melt curve analysis compatibility	Yes	Only for annealing probes (e.g. molecular beacons). Hydrolysis-probes (e.g. TaqMan) do not allow melt curve
Versatility	Readily adaptable. Any new target can be included in an experiment with a new primer pair	Every target requires a specific probe(s) in addition to primers. Probe and primer must have similar parameters.
Recommended Applications	Gene expression analysis, Species diversity analysis (16S or similar), HRM analysis, Similar applications...	Diagnostic qPCRs, Genotyping/allele abundance, Other applications requiring high specificity.



3. Target choice, primer and probe design

3.1 Target

The target region should be chosen to maximise the efficiency and specificity of the qPCR reaction and should generate unambiguous results that are biologically meaningful.

Briefly:

- A target region should be unique within the template sample. In other words, it should have low homology with other regions in the genome or transcriptome of the host organism or other contaminating organisms in the sample.
- Target GC content should be ideally between 30-70%.

If a transcript target is known to have multiple splice variants, the target region should be chosen to span differentiating exon splice junctions unless no distinction among variants is desired. This allows the different splice variants to be detected based on differing amplicon lengths, which can be assessed post qPCR or assessed by standard PCR using the same primer pair prior to running a qPCR.

In the case of highly similar transcripts or genes, targets should be chosen such that primers are in the most highly varied regions and their sequence terminating with a differentiating nucleotide. This could be in the 5' or 3' UTRs for highly similar transcripts or introns for highly similar gDNA targets.

3.2 Primer design

Primers should be designed to avoid or minimise secondary structures (hairpins, self-dimers, or cross-dimers). This is important both for intercalating dye-based qPCR detection (all dsDNA structures will generate signal, and secondary structures will introduce artefacts in the analysis) and for probe-based qPCR detection (primers will become limiting reagents in the reaction, reducing the efficiency and the sensitivity). Indeed, avoiding secondary structures is important to maximize reaction efficiency. Suitable secondary structure prediction software should always be used for optimal design. Tools are available online, including a free online version of the commercial Beacon primer design program[3] and Primer3[4]. Primer BLAST against genome and transcriptome databases should be conducted with multiple suitable primer pairs, in order to identify the best set or primers possible and to ensure high specificity.

In addition to these general considerations, further design features are:

- Primers should be 18-25bp in length
- Primers should not have a Tm difference of more than 2˚C, to ensure annealing temperature is optimal for both primers.
- Choose primer sequences to have one or two G or C residues at the 3' end, known as a 3'-anchor.
- Primers should be designed such that amplicon length is between 80-250 bp and ideally at the shorter end of this range. For the qPCR to give reliable data, PCR efficiency should be as close to 100% (indicating perfect doubling of the target) as possible, and amplification efficiency is inversely correlated to target length.
- When designing primers to detect eukaryotic gene transcripts (cDNA), it is good practice to choose primers that span exon splice junctions. This ensures that any contaminating genomic DNA will not be amplified during qPCR, since it contains the un-excised intron between the 2 exons.
- When designing primers for a new target, choose 2 to 3 primer pairs to test out in the lab.
- Primers should be added to reactions at the amount recommended by the qPCR mix manufacturer. Final concentration for each primer usually ranges between 300-900 nM and can be varied within this range to optimise reactions.

3.3 Probe design

Considerations for designing oligonucleotide probes are the same as those for primers, with some additional parameters.

- Probe sequences should be as specific as possible and not cross-react with any off-target regions in the template sample or with primers.
- The annealing temperature of the probe should be 5-10 ˚C higher than that of the amplification primers because the probe must remain bound to the template during amplification.
- The sequence should have 30-80% GC content and should be designed to contain more Cs than Gs, as guanine residues can quench fluorophores.

- Critically, 5'G residues should be avoided during probe design. The fluorophore remains attached to this residue even after hydrolysis and this will cause signal quenching after probe degradation by DNA polymerase.
- Probes should be of good quality and high purity (e.g., HPLC purification or comparable). When setting up your laboratory for probe-based qPCR experiments for the first time, you should trial different suppliers and purification methods to identify the best suited to your application.
- The fluorophore and the quencher molecule used should be compatible (Table 2). The chosen fluorophore(s) must be detectable by the qPCR instrument in use. The FAM dye is the most broadly compatible fluorophore and is widely available from most suppliers. Thus, it is a good choice for single-plex probes. If designing probes for multiplex reactions, see tips in the Multiplex qPCR Design section 3.4 below.
- Final concentration of an individual probe in a reaction should be between 100-500 nM, starting at the amount recommended by your qPCR mix manufacturer. Vary from that if necessary to optimise a reaction.

Table 2: Fluorescent dye and quencher compatibility

Dye	Emission wavelength (nm)	Quencher
FAM	520	ZEN™-Iowa Black® FQ, Black Hole Quencher®-1
ATTO™ 488	522	
TET	539	
ATTO™ 532	554	
JOE	555	
HEX	555	
TYE™ 563	563	Iowa Black® FQ, Black Hole Quencher®-2
Cy®3	564	
ATTO™ 550	575	
TAMRA	583	
ATTO™ 565	591	
ROX	608	
ATTO™ Rho 101	609	
TEX 615	613	
Texas Red®-X	617	
TYE™665	665	
Cy®5	668	

3.4 Multiplex qPCR design

Multiplex qPCR reactions offer additional benefits to probe-based detection. They allow the quantification of multiple targets in the same reaction using probes with distinct fluorophores.

Benefits of multiplexing qPCR:

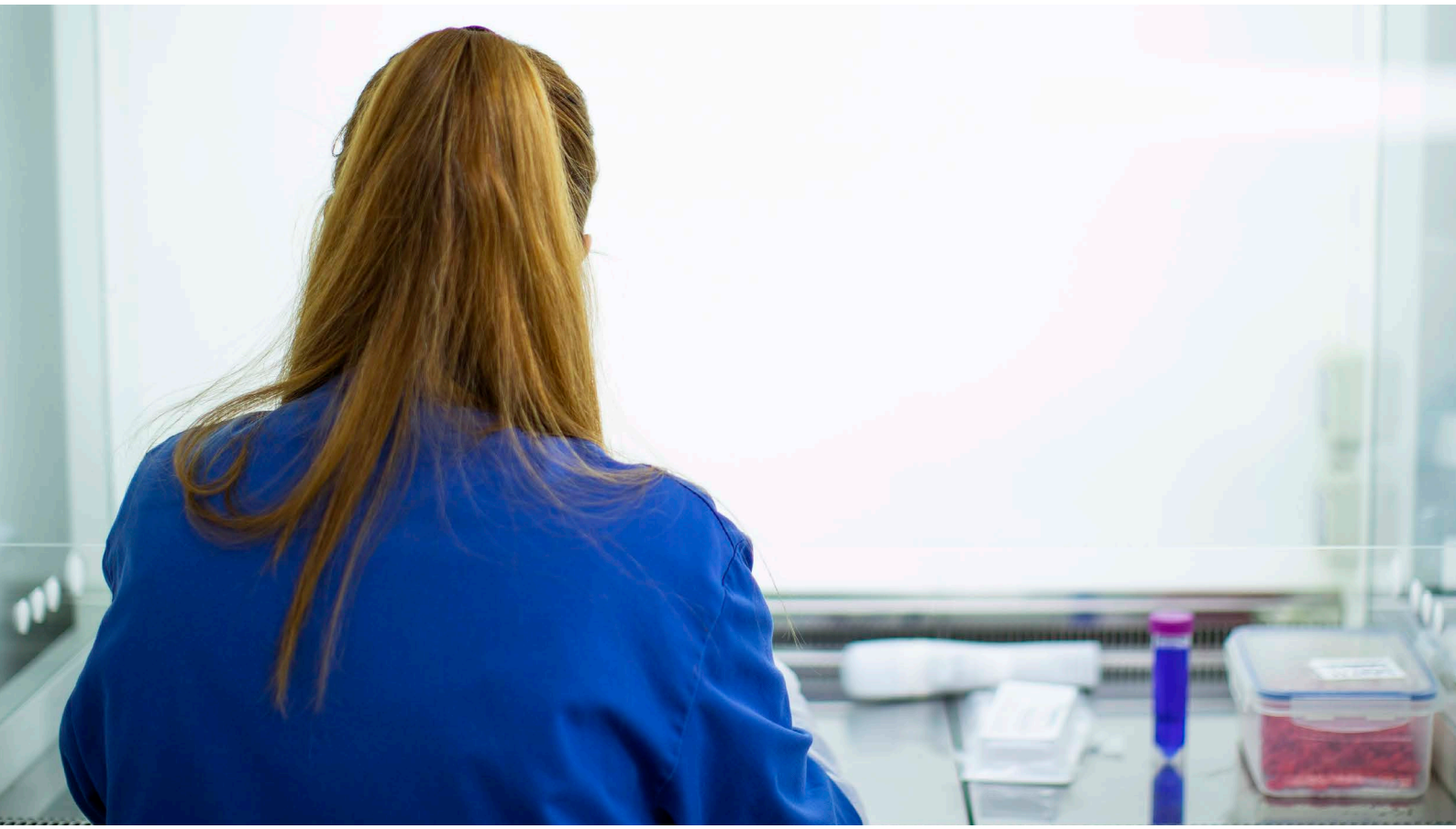
- Increased experimental throughput, because multiple targets can be quantified in one reaction.
- Reduced pipetting error when the normaliser gene is assayed in the same reaction as the targets.
- Reduced sample consumption because more information can be extracted from a single reaction.

However, multiplexing also has challenges that should be minimised with careful experimental setup. The considerations described for primer and probe design, and target choice in singleplex apply to multiplex qPCR.

Considerations for multiplex primer design:

- Targets and primers with similar efficiencies should be chosen, so that the more efficient targets do not consume reagents and further reduce the efficiency of others primer-probe sets.
- Primers and probes should not form cross dimers among the different sets.
- Extra care should be taken so that the fluorophores chosen for each probe are spectrally distinct.

To evaluate individual target abundance, each probe and primer set should be tested for efficiency in single-plex assays and then in multiplex. Thus, if one target is more than 3 Cq different to other targets and if efficiency for a specific set is reduced in multiplex assays, steps to optimise the compromised reaction should be taken. Alternatively, additional primers or targets should be considered.



4. Primer validation and reaction efficiency calculation

Key points for primer validation

1. Ensure primers are specific, i.e., they amplify the expected unique target in the chosen sample type and do not generate unexpected amplicons.
2. Ensure primers do not produce dimers (this should be validated empirically as well as *in silico* during primer design).
3. Calculate primer reaction efficiency in qPCR and ensure it is between 90-110%.

Validation of primers should be carried out on a sample of the same type as will be used in experiments. For instance, gDNA samples should be used if genomic DNA is to be tested, and reverse transcribed RNA if cDNA samples are to be used. Primers should be tested in a standard PCR or qPCR with an annealing temperature gradient around the predicted primer pair Tm to assess the range of temperatures that can be used without introducing amplification artefacts.

Upon completion, the reactions should be run on an agarose gel even if melt curve analysis has been conducted in a test qPCR. Potential artefacts include low molecular weight, short-length (<100 bp) products caused by primer dimers and hairpins and longer amplicons resulting from non-specific target amplification because of mis-priming. If artefacts are visible, the annealing temperature range should be increased to the maximum possible and the number of cycles decreased to a maximum of 35-38 cycles. If artefacts still occur, after reviewing sample preparation (e.g., no residual DNA is present in RNA samples, or sample contamination has not occurred), new primers should be designed for a different region of the target.

Using purified target or target cloned into plasmid vectors is not recommended when testing primers, because an obvious source of artefacts is mis-priming or off-target priming of non-target nucleic acids present in experimental samples. This information is missed if a purified target is used for validation. However, purified targets are useful as reaction positive controls.

Reaction efficiency is calculated by carrying out a qPCR on a dilution series of a sample with a known amount of the target, plotting the logarithm of the amounts of target against the observed Cq for each sample, and curve-fitting a linear equation through the resulting data points. This generates data similar to that shown in Figure 8.

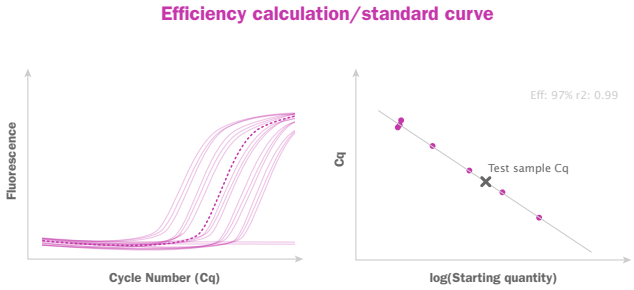


Figure 8

Standard curve amplification plots and resulting efficiency curve

Calculating qPCR efficiency

Plot the logarithm of the amounts of target against the observed Cq for each reference sample. Calculate the slope of the best fitting line:

$$\text{Slope} = \Delta C_q / \Delta \log(\text{sample amount})$$

is used to calculate the reaction efficiency for the primer pair during the linear portion of logarithmic amplification in the qPCR using the equation:

$$\text{Efficiency (E)} = 10^{(-1/\text{slope})}-1$$

As discussed, efficiency for a primer pair should be between 90-110%. An efficiency below 100% reflects the biochemical reality that perfect doubling of a target sequence at each cycle rarely occurs under normal conditions. Efficiencies above 100% are usually observed when reaction inhibitors are present and alter efficiency of each dilution to a different degree.

Additional parameters calculated from the standard curve are the correlation coefficient (R2), which indicates how well the curve fits the data points and which ideally should be 1 (or 100%) indicating a perfect fit, and the Y-intercept that theoretically predicts the detection limit of the qPCR with that primer pair (Figure 8).

5. Quantification methods in qPCR

As the name implies, qPCR allows quantification of nucleic acid targets with a known sequence. Depending on how a qPCR experiment is set up it can quantify both DNA and RNA targets and can yield quantitative information in absolute units, units of mass, or moles (copy number) of template molecule. Alternatively, qPCR can also yield relative quantitative data, where the measurement units reflect the amount of target molecule relative to one or more other standard molecules in the same sample, and/or relative to target abundance present in a reference sample.

Both approaches utilise the C_q values in calculating biologically meaningful information. However, it is critical that all comparisons and statistical analyses on qPCR data should be done on the units derived from one of the above quantification methods, not the C_q values themselves, as these are just the instrument output and can lead to erroneous conclusions when compared directly. Additionally, C_q values reflect differences on a logarithmic scale and one C_q difference reflects a 2-fold difference in actual target abundance. Thus, small C_q differences among samples may still reflect huge differences in the target amount.

We present each quantification approach below in detail, along with their respective strengths and weaknesses.

5.1 Absolute quantification

To achieve absolute quantification of a target molecule with qPCR, a series of standard reference samples containing known amounts of the target molecule are necessary. The standard sample can be an experimental sample in which target abundance has been estimated in prior experiments, or it can be purified target molecule (that is generated through PCR and gel extraction or cloned into a vector). Although the latter approach is not recommended, as discussed previously.

Regardless of type, the standard sample is used to generate a dilution series with enough data points to span biologically relevant target abundances down to non-detectable levels. These samples are then included in a qPCR experiment along with the experimental samples under evaluation. The standard sample dilution series is then used to form a standard curve as described previously for reaction efficiency calculations. The linear equation that best fits the standard curve is then used to estimate the amount of target present in experimental samples based on their experimentally observed C_q (Figure 8).

Benefits and shortcomings of absolute quantification

Benefits

The benefit of this approach is that it allows unambiguous comparison of data among different experiments, whether within the same lab or between different research groups. It also enables the accurate quantification of target abundance within individual cells and thus gives a more thorough view of the system under study.

Shortcomings

Conversely, the biggest drawbacks to using absolute quantification are (i) the increased time necessary to generate a standard sample (purified or cloned) and to run the standard curve and (ii) the corresponding increase in costs for each target template being evaluated. This also means that if many targets are being assessed in parallel, experimental throughput is reduced.

This approach is therefore best suited for when the absolute value of one or two target molecules are necessary for the experiment. Conversely if multiple targets are being assessed across many samples the efficiency of this approach is greatly reduced.

5.2 Relative quantification

In this approach, qPCR is used to estimate the abundance of a target molecule relative to a reference molecule (or molecules). Relative quantification is primarily used in gene expression studies but can be expanded to other applications when the criteria described below are met. The rationale of this method is based on the empirical observation that certain genes in any organism maintain stable expression levels under most experimental conditions, and therefore their transcript abundance does not fluctuate significantly after experimental treatments. These are known as housekeeping genes and are well suited as reference genes because their abundance is similar across all samples in an experiment.

To achieve relative quantification, the C_q values of both the target and reference genes are measured by qPCR for every sample in an experiment and the abundance of the target gene is calculated as a proportion of the reference gene's expression (Figure 9).

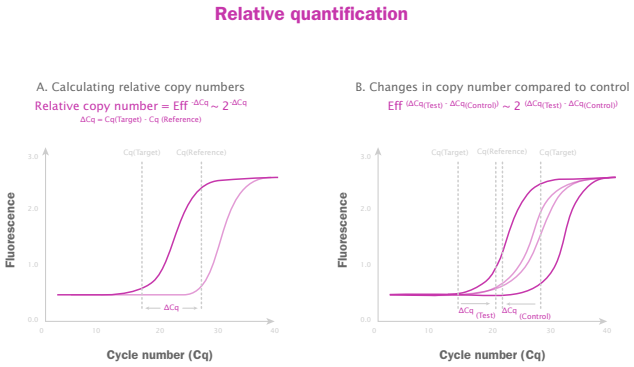


Figure 9

Relative quantification using a reference gene in one sample (A) and in a test versus a control sample (B)

This value provides no information on the absolute number of target molecules, but it does allow target abundance to be compared among samples, as it is normalised to that of the reference gene, which is similar across samples. To acquire meaningful experimental data with this approach, it is critical to ensure that the selected reference genes are indeed stable across all conditions and treatments used in a particular experiment. This must be validated through prior experiments, established from transcriptomic datasets, or validated by absolute quantification using a standard curve. Some common reference genes in mammals include: *ACTB* (actin B), *GAPDH* (glyceraldehyde phosphate dehydrogenase), and *18S rRNA* genes[5]. A list of mammalian reference genes can be found online[6]. In plants common reference genes are: ubiquitin ligases (UBQ10 in Arabidopsis, UBQ1 in legumes, TBC (tubulin C), EF1a and PP2A (protein phosphatase 2 A)7. Other genes not listed here may be suitable for different organisms, tissue types or experimental conditions.

Although reference genes are the most used means of normalisation among samples, it is also possible to use other stable parameters in a relative quantification experiment. These can be sample mass, total nucleic acid input in the qPCR, or similar sample traits that are unchanged under the chosen experimental conditions and introduce minimal experimental error or bias.

Key benefits and shortcomings of relative quantification

Benefits

The key benefit of using relative quantification in qPCR is that it allows the high throughput analysis of multiple targets in parallel, with minimal additional cost in reagents and time used for experimental setup. All that is needed to include a new target in an experiment is a validated primer pair. The same reference gene(s) can be used to assess the expression of many target genes, after verification that their expression level is stable under the treatment being studied. On the contrary, absolute quantification requires a full standard curve for every target being examined.

Shortcomings

Drawbacks of relative quantification include the fact it is relative, and therefore comparison of expression values between different experiments and among different lab groups or publications are risky. Relative quantification experiments on the same target gene that use different reference genes are also not directly comparable. Additionally, although it is highly accurate, this approach carries the potential bias of unexpected fluctuations in reference gene expression levels, even after validation. Finally, because the data generated is relative, it is only meaningful in the context of gene expression levels and cannot be used as a stand-alone value on the actual target abundance.

6. Experimental setup, data analysis and MIQE

6.1 Setting up qPCR experiments

There are numerous steps you should follow to ensure you make the most efficient use of qPCR in order to generate biologically meaningful data[8].

1. Consider which targets you will include in your analysis.
2. Enumerate the experimental conditions, treatments and time points you will need.
3. If conducting absolute quantification, establish you have a reference sample with a known quantity of the target(s) you are studying. If doing relative quantification, identify suitable reference genes that are stable under the conditions studied. If reference genes are not available, consider spiking samples with an exogenous reference target in proportion to the amount of sample added.
4. Design and validate primers for targets and reference genes or use primers that have been characterised in the literature. In the latter case you should still test primer efficiency and specificity, and ensure they work as reported in your hands.
5. Calculate the number of biological (number of different individuals, cell cultures, or colonies) and technical (number of reactions with the same template sample) replicates you will use for each experimental condition. If necessary, use a statistical power test to establish the minimum number of biological replicates you need to identify statistically significant changes in target expression. The absolute minimum of biological replicates is 3, and this is commonly reported in the literature, but this is by no means sufficient to guarantee a representative and biologically meaningful result and should not be blindly chosen as adequate. The number of technical replicates is usually 2 to 3 but can be more, if you suspect a source of strong technical variation. If conducting an absolute quantification experiment don't forget to include standard curve samples. Usually, a standard sample dilution series ranges from 10^8 down to 10^0 target molecule copies and includes a no-template control (NTC). However, the upper range can be empirically estimated and altered, as necessary.
6. Identify the necessary control samples. Include a positive control sample to test reaction mix activity. No template controls (NTCs) should be included for every primer pair and run on every plate. If running multiple plates for the same experiment, consider what positive control samples/targets you can use as intra-plate controls, to minimise intra-plate variation during analysis. If using cDNA samples, include no reverse transcription controls (NRT) to calculate the contribution of contaminating gDNA or other templates that can skew results.
7. Choose samples that will provide the most accurate biological result. For example, bulk extraction of nucleic acids from whole organs or organisms may mask transcriptional changes at local or tissue level. Dissection or selection of specific cell and tissue types may reveal localized differences in target levels. If possible, harvest samples under the same conditions, flash freeze and store at < -70 ˚C until extraction.
8. Ensure sample handling and preparation is optimal and results in samples of the highest quality possible. RNA is particularly prone to degradation, and this can severely impact gene expression or diagnostic analyses. DNA is more stable, but care should be taken to ensure samples are protected. Select a purification method that gives high purity nucleic acids with an $A_{260}/A_{280} > 1.8$ and an $A_{260}/A_{230} > 2.0$. Ensure integrity of purified samples by agarose gel electrophoresis or capillary flow analysis (*e.g.*, RIN Bioanalyzer). RNA samples should be treated with DNase to remove contaminating DNA and the absence of DNA should be confirmed by endpoint PCR and or the inclusion of NRT controls in downstream qPCR. Quantify total DNA or RNA using the same method for all samples. Do not use values generated by different methods (*e.g.*, do not compare UV absorbance to dye-based quantification results within the same experiment).
9. Once you are ready to set up your qPCR plate, ensure you minimise sources of contamination and technical error. If possible, work in a clean bench or at least in a dedicated area of your lab. Use a liquid handling robot if available. Prepare mastermixes for each primer set (and probe if applicable), ensuring there is a surplus of mastermix to that necessary for all samples.
10. When combining samples with the qPCR mastermix it is ideal to use large volumes of sample. Commonly used sample volumes are 10-20% of a qPCR reaction. This works out from 2 to 4 µL in a 20 µL total reaction volume. If possible, prepare your mixes and sample

dilutions so that they are mixed at roughly a 60-40% mastermix to sample volume. This ensures you can use the same pipette at the midrange of its pipetting volume for setup, eliminating as much pipetting error as possible. This also ensures better mixing of mastermix and sample. Make sure wells are closed and sealed properly, spin down the plate in an appropriate centrifuge and make sure there are no air bubbles present in the wells, which will cause fluorescence aberrations during thermocycling.

11. Run the thermocycling program as recommended by the product manual and choose an annealing temperature that you've established as optimal when validating primers for the experiment. If appropriate, include a melt curve step at the end of the thermocycling product. Exact settings for melt curve measurements vary by instrument but are usually supplied as pre-set in the accompanying software. Refer to the corresponding manual.
12. Post-acquisition, evaluate the results for obvious issues with amplification plots and melt curves, or the presence of amplicons in the NTC and NRT controls. Make note of any such occurrences prior to analysis. If you do discover issues at this point, refer to the "Troubleshooting" section to find appropriate solutions. Generally, product accumulating in the NTC at 5 or more cycles after all test samples is considered acceptable and analysis can be carried out normally. However, it is good to verify that such products are distinct from the target and are not present in the test samples. Most of the times they are just primer dimers.

6.2 Analysis and peer-review quality data reporting

Assuming there are no obvious issues with the qPCR run (see point 11 above) you can export and analyse the run data. Most qPCR instruments will automatically define the threshold and generate C_q values for every sample. This threshold can be manually adjusted to higher or lower values, but this is not recommended for inexperienced users as it can lead to erroneous C_q values. Establish an upper C_q value above which you will exclude samples from the analysis: C_q values above 35-40 cycles correspond to very low input template amounts (<10 target copies at the start of the reaction). At such concentrations, amplification during initial qPCR cycles is mostly stochastic and increases technical variation considerably. Longer programs with many cycles also introduce a higher number of artefacts, primer dimers, mis-priming products and incomplete target fragments, thus higher C_q values may not always reflect true target quantity. Generally, C_q values of >40 are questionable, but should be carefully evaluated for target specificity prior to inclusion in or exclusion from the analysis. Depending on whether you are conducting absolute or relative quantification the exact calculations will change. We outline steps for each method below.

6.3 Absolute quantification data analysis

In absolute quantification, each technical replicate C_q will be compared to the standard curve and from that the input template amount can be calculated. Most modern software will automatically generate a standard curve, perform calculations, log-transformation, and export initial sample quantities in relevant units (ng, copies, or mols per unit of volume). Because such units are on a linear scale you can directly calculate descriptive statistics for biological replicates and run statistical analysis directly using the chosen abundance units.

However, should you wish to conduct the analysis yourself on exported C_q data, you should take the following steps:

1. Plot the standard curve using the exported C_q values versus the $\log_{10}$- value of each reference sample dilution's starting quantity.
2. Find the best-fitting line to your data. This can be done with various programs, including Microsoft Excel, R, or Python scripts. Retrieve the equation describing this best fitting line and note its slope and Y-intercept.
3. Calculate the mean value of technical replicates for each experimental sample.
4. Use the standard curve equation generated in step 2 to input the mean C_q of each experimental sample from step 3 and calculate the $\log_{10}$ starting quantity for each sample.
5. Exponentiate 10 to the $\log_{10}$ starting quantity calculated in step 4 to derive actual experimental sample starting quantities.
6. Calculate descriptive statistics and run statistical analysis on actual starting quantities to draw experimental conclusions.

6.4 Relative quantification data analysis

In the **ΔC_q method**, the difference in target and reference gene(s) C_qs is calculated according to the equation (1):

$$\Delta C_q = C_q(\text{Target}) - C_q(\text{Reference}) \quad (1)$$

And the relative expression of a target gene is calculated by

$$E^{-\Delta Cq} \quad (2)$$

with C_q(Target) and C_q(Reference) being the experimental Cq values obtained by qPCR for the target and reference genes, respectively, in the same sample, and E is the reaction efficiency. In an ideal scenario, where reaction efficiency is 100% for both target and reference genes, E = 2. This was the approach originally used by Livak and Schmittigen and can still offer valuable experimental information when experimental efficiency is high[9]. It has, however, been criticised as inaccurate when reaction efficiency is much lower than 100%.

The experimentally calculated efficiency can also be used in equation (1) but only if it is the same or very similar for both target and reference genes. An alternative to experimentally calculated efficiency (via standard curve) is to mathematically calculate the efficiency of each reaction (by linear regression analysis of Cq data versus log-fluorescence data of every sample's amplification plot). Free software for such reaction efficiency calculation is available[10] and is also a component of some commercial software packages provided with qPCR instruments. This calculated efficiency can be used as the value of E in equation (1). Assuming reaction efficiency (whether theoretically or experimentally calculated) is comparable for both target and reference, the average efficiency can be used as the E value in equation (1). This approach should not be used when calculated efficiencies for target and reference molecules are greatly different. Regardless of what method is used, the relative expression values generated allow the comparison of a target's expression across samples as well as to that of other targets normalised to the same reference gene. It is thus an extremely versatile tool for conducting gene expression analyses with multiple targets.

The **$\Delta\Delta C_q$ method** uses a sample for normalisation in addition to reference genes. To do so the ΔC_q value is calculated for every sample, as per equation (1), and subsequently the ΔC_q of each test sample is normalised to that of the reference/normaliser sample according to equation (3) below:

$$\Delta\Delta C_q = \Delta C_q(\text{Test}) - \Delta C_q(\text{Control}) \quad (3)$$

And the fold-change in gene expression compared to the control sample is given by:

$$E^{-\Delta\Delta Cq} \quad (4)$$

where ΔC_q(Test) and ΔC_q(Control) are, respectively, the ΔC_q values of the test samples and the control or reference sample to which all others are normalised. E is again the average calculated efficiency of both target and reference genes. This can also be written as:

$$2^{-\Delta\Delta Cq} \quad (5)$$

when assuming perfect efficiency for both reference and target reactions. This method gives the relative expression values of the target gene in all samples as the fold-change compared to the selected control sample, which will always have a fold-change value of 1[11]. In an experiment this will usually be an untreated or unmodified genotype sample. This value of a target's relative quantity is comparable to that of the same target in all samples across an experiment, but is not comparable among different targets, because every target has a value of one in the control sample. Thus, the additional level of normalisation may allow a more robust estimate of a single target's changes across an experiment but does not allow any inferences to be made on the relative quantities of different targets even within the same sample.

The following steps should be taken when conducting relative quantification:

1. Calculate reaction efficiencies and the mean of all technical replicate C_qs for every target in each biological sample. These mean values should be used for all subsequent steps.
2. Calculate the mean C_q of all reference genes in each biological replicate and use as the reference gene C_q. This can be used to calculate the ΔC_q for all target genes in that sample according to equation (1).
3. The normalised relative expression of each target in all biological samples can then be calculated according to equation (2).
4. For ΔC_q expression values, calculate the mean of all biological replicate normalised expression values for a given experimental treatment. This value is the mean target gene normalised expression level for that treatment.
5. If using the $\Delta\Delta C_q$ method, calculate the ΔC_q values from equation (1) for every test sample. Calculate the mean of all biological replicates in the control condition.
6. Use this mean relative expression of the control condition to normalise expression in all test conditions by calculating the $\Delta\Delta C_q$ value for every biological replicate, according to equation (3).
7. Use equation (5) to calculate fold-change compared to the control sample mean. Also calculate the fold-change for each biological replicate in the control treatment to get individual control sample expression. Expression for all the control samples should be close to 1. Log_2-transform fold-change expression values of all biological samples to calculate standard deviation, standard error, and confidence intervals as well as run statistical analysis.

Regardless of the quantification method you choose, the statistical analysis method should be decided during experimental setup and not after the experiment is run. A statistical power test should also be run at this stage to ensure sufficient replication in an experiment, so that resulting conclusions are meaningful and representative of actual biological events. Normality and skewness tests should be run on log-transformed expression data to ensure it is normally distributed, prior to conducting any statistical analysis. If data is not normally distributed even after log-transformation, consider non-parametric tests. For further information on appropriate statistical tests and testing requirements consult a statistics or statistics for biologist's textbook or website.

6.5 Data reporting

Prior to conducting experiments, it is important to be aware of the minimal amount of experimental information needed to publish qPCR data in peer-reviewed journals. A set of guidelines to provide the minimum information for publication of quantitative real-time PCR experiments (MIQE) has been published and is widely accepted in the scientific community[12]. Although not mandatory, is it good practice to adopt these guidelines regardless of whether you choose to publish or not. This ensures greater reproducibility of results, a higher quality of data and clarity in communicating your experiments to other researchers because of standardisation. You can find a checklist of the MIQE criteria in the table below.

Table 3: MIQE reporting criteria checklist, (E: Essential, D: Desirable to report this information)

MIQE reporting checklist
Experimental design
▪ Defined experimental and control groups (E)
▪ Number of individuals and technical replicates per group (E)
▪ Who carried out the assay? (D)
▪ Acknowledge author's contributions (D)
Sample
▪ Description (E)
▪ Volume/mass of sample processed (D)
▪ Micro or macrodissection (E)
▪ Processing procedure (E)
▪ If frozen, how and how quickly? (E)
▪ If fixed, with what and how quickly (E)
▪ Sample storage conditions and duration (especially FFPE[1] samples) (E)
Nucleic acid extraction
▪ Procedure and/or instrumentation (E)
▪ Name of kit and details of any modifications (E)
▪ Source of additional reagents used (D)
▪ Details of DNase or RNase treatment (E)
▪ Contamination assessment (DNA or RNA) (E)
▪ Nucleic acid quantification (E)
▪ Instrument and method (E)
▪ Purity (A260/A280, optional A260/A230) (D)
▪ Yield (D)
▪ RNA integrity: method/instrument (E)
▪ RIN/RQ or Cq of 3' and 5' transcripts (E)
▪ Electrophoresis traces (D)
▪ Inhibition testing (Cq dilutions, spike or other) (E)
Reverse transcription
▪ Complete reaction conditions (E)
▪ Amount of RNA and reaction volume (E)
▪ Priming oligonucleotide (if using a gene specific primer and concentration (E)
▪ Reverse transcriptase and concentration (E)
▪ Temperature and time (E)
▪ Manufacturer of reagents and catalogue numbers (D)
▪ C_qs with and without reverse transcriptase (D)
▪ Storage conditions of cDNA (D)
qPCR target information
▪ Gene symbol (E)
▪ Sequence accession number (E)
▪ Location of amplicon (D)
▪ Amplicon length (E)
▪ *In silico* specificity screen (BLAST, etc.) (E)
▪ Pseudogenes, retropseudogenes, or other homologs? (D)
▪ Sequence alignment (D)
▪ Secondary structure analysis of amplicon (D)
▪ Location of each primer by exon or intron (if applicable) (E)
▪ What splice variants are targeted? (E)

MIQE reporting checklist
qPCR oligonucleotides
▪ Primer sequences (E)
▪ RTPrimerDB identification number (D)
▪ Probe sequences (D)
▪ Location and identity of any modifications (E)
▪ Manufacturer of oligonucleotides (D)
▪ Purification method (D)
qPCR Protocol
▪ qPCR Protocol
▪ Complete reaction conditions (E)
▪ Reaction volume and amount of cDNA/DNA (E)
▪ Primer, (probe), Mg^{2+}, and dNTP concentrations (E)
▪ Polymerase identity and concentration (E)
▪ Buffer/kit identity and manufacturer (E)
▪ Exact chemical composition of the buffer (D)
▪ Additives (SyGreen, DMSO, and so forth) (E)
▪ Manufacturer of plates/tubes and catalogue number (D)
▪ Complete thermocycling parameters (E)
▪ Reaction setup (manual/robotic) (D)
▪ Manufacturer of qPCR instrument (E)
qPCR Validation
▪ Evidence of optimisation (from gradients) (D)
▪ Specificity (gel, sequence, melt, digest) (E)
▪ For SyGreen, C_q of the NTC (E)
▪ Calibration curves with slope and y intercept (E)
▪ PCR efficiency calculated from slope (E)
▪ Confidence intervals for PCR efficiency or SE (D)
▪ R2 of calibration curve (E)
▪ Linear dynamic range (E)
▪ C_q variation at limit of detection (E)
▪ CIs throughout range (D)
▪ Evidence for LOD (E)
▪ If multiplex, efficiency and LOD of each assay (E)
Data analysis
▪ qPCR analysis program (source, version) (E)
▪ Method of C_q determination (E)
▪ Outlier identification and disposition (E)
▪ Results for NTCs (E)
▪ Justification of number and choice of reference genes (E)
▪ Description of normalisation method (E)
▪ Number and concordance of biological replicates (D)
▪ Number and stage (reverse transcription or qPCR) of technical replicates (E)
▪ Repeatability (intra-assay variation) (E)
▪ Reproducibility (inter-assay variation, CV) (E)
▪ Power analysis (D)
▪ Statistical methods for results significance (E)
▪ Software (source, version) (E)
▪ Cq or raw data submission with RDML (D)

[1]FFPE: Formalin-Fixed Paraffin Embedded tissue

7. Applications

Applications of qPCR include the quantification of DNA molecules and various species of RNA. We summarise the most common techniques below.

7.1 1-Step RT-qPCR and 2-Step RT-qPCR

This variant of qPCR focuses on quantification of RNA molecules. Because PCR works on double stranded DNA templates, quantifying RNA molecules requires their conversion to DNA first. This is achieved using enzymes known as reverse transcriptases, or RTases, which allow the assembly of single-strand complementary DNA (cDNA) from an RNA template. This is known as reverse transcription and, when coupled to qPCR it gives rise to reverse transcription-qPCR or RT-qPCR. Traditionally, RT-qPCR is completed in two steps. A first step in which RNA is converted to cDNA and a second step where the prepared cDNA is used as template in a qPCR. This allows for efficient use of RNA samples, because usually only a portion of purified RNA is used in cDNA synthesis and a cDNA preparation can be used in multiple qPCRs to detect multiple different targets or in other applications, such as transcript cloning or library construction.

For reverse transcriptases to work, they require primers like DNA polymerases. Three different types or primers or combinations thereof are generally used.

- Oligo(dT) primers are short oligonucleotides (15-20 nucleotides long) comprising solely of thymidine residues and bind indiscriminately to all poly-A containing RNA molecules. The use of oligo(dT) primers alone generally leads to 3'-end enriched cDNA, because the poly-A sequences are present at the 3' of eukaryotic mRNAs and the efficiency of first strand cDNA synthesis reduces proportionally to target length. These primers are not suitable for prokaryotic cDNA synthesis, because prokaryotic RNA is not polyadenylated.
- Random hexamer primers are oligomers composed of 6 nucleotides or multiples thereof containing random nucleotide residues. These bind to any RNA molecule with high enough complementarity, but with less efficiency compared to fully complementary primers.
- Gene-specific primers target one specific sequence, as such, they enable efficient cDNA synthesis of only the target molecule. This necessarily means the cDNA is suitable for amplification only of this one target.

Note:

For unbiased yet efficient cDNA synthesis, it is usual to use a mix of random hexamers and oligo-dT primers in general applications that assess multiple targets, whereas gene specific primers are used when sensitive unique target amplification (as is the case in diagnostic qPCRs) is required.

In 1-step RT-qPCR, an RNA sample and target-specific primers are added to a qPCR mix containing both a reverse transcriptase and a DNA polymerase. Thus, first strand cDNA synthesis and qPCR are carried out in a single combined cycling program.

In this approach, only the target(s) for which primers were included in the reaction mix are converted to cDNA and amplified. This reduces the number of sample handling steps and experimental time but sacrifices flexibility of sample use. However, if used incorrectly, without proper reaction efficiency calculations, it is susceptible to experimental errors in target quantification, because the efficiency of the RT reaction with a given primer set may differ to amplification efficiency in a qPCR. Such primer-specific bias of cDNA synthesis is less of an issue in 2-step RT-qPCR when using oligo-dT primers mixed with random hexamers, which indiscriminately prime all RNA molecules within a sample.

Conversely, the use of target-specific primers may benefit low-abundance target amplification, making the overall process more sensitive. 1-step RT-qPCR does offer significant advantages and can greatly enhance research workflows. Choosing between 1- or 2-step RT-qPCR depends on the number and type of targets being investigated and what other applications the same samples might be used for.

7.2 Gene expression

One of the key-uses of RT-qPCR is gene expression analysis. This method allows quantification of the number of transcript molecules (mRNA) that are produced by a certain gene in a particular organism, tissue or cell type. In most cases, gene expression is evaluated by relative quantification through RT-qPCR using one or more housekeeping genes as reference molecules. The experiment is then set up to compare the expression of the target(s) among the different experimental conditions after standardising to the reference molecule(s). This normalisation and relative quantification is usually done using either the delta C_q (ΔC_q) or the delta delta C_q ($\Delta\Delta C_q$) method. As seen above, both these methods rely on qPCR to

acquire C_q values for the reference and target genes and are compatible with dye-based and probe-based detection.

A critical factor in qPCR relative quantification is the selection of reference genes that are appropriate for the organism and experiment. Multiple peer-reviewed publications have shown that reliance on one reference gene is risky and potentially leads to erroneous conclusions on gene expression. This is because no gene's transcript remains at constant levels under every possible experimental condition, and this includes so-called housekeeping genes, which may maintain their transcripts at or close to a fixed level, but still fluctuate significantly under various experimental conditions. To avoid this issue, it is recommended you use two or more reference genes for normalisation and these genes should be validated as stably expressed under all the conditions that will be used in a qPCR experiment. Validation can be based on published data, freely accessible transcriptome databases specific to the organism of interest, or experimentally by carrying qPCR for absolute quantification of the reference genes, or by establishing a strong correlation between reference gene C_q and total input RNA in the reaction.

Regardless of which method of calculating you choose for your gene expression analysis, most commercial software packages, and many open-source packages online will carry out these analyses automatically, provided samples and reference genes are correctly input in the software.

7.3 Species/allele abundance

qPCR is also routinely used to quantify the abundance of various alleles in an organism's genomic DNA, or to quantify various species-specific genes in crude biological samples comprising more than one organism. The latter is often used as a proxy for species abundance in the same sample. For such analyses, absolute quantification is usually chosen, as it provides information that is comparable across sample types and studies. Although, it is also possible to use relative quantification, by normalising to sample mass, cell number or total DNA input in a reaction[12]. For species abundance studies, one or more genes that are uniquely specific for each strain or species being evaluated are chosen as targets. Frequently, these will be 16S or 18S rRNA encoding genes for prokaryotic and eukaryotic species, respectively, or the internal transcribed sequences (ITS) intervening between the small and large rRNA subunit genes. Different targets may be chosen as is appropriate for the planned study.

Regardless of the target, an absolute quantification experiment is carried out as described in section 5.1 using a standard curve for the selected target gene(s). Reference sample dilutions for the standard curve should be chosen to span the entire range of biologically relevant levels of target. Sample inputs should also be carefully measured and used to normalise the absolute quantities measured, because contrary to relative quantification methods, it is rare to find a common internal control that can account for handling errors or varied purification efficiency among such samples. Where possible, it is good practice to include targets that do not vary in abundance across experimental conditions, which can act as an internal amplification control. However, this is not always possible. Statistical analysis should be carried out directly on calculated abundances without log-transformation unless the data fails normality and skewness testing.

7.4 Diagnostics

Absolute quantification by qPCR is a powerful tool for diagnostics, as it allows the simultaneous detection and quantification of various pathogens, or disease-related markers, and thus provides inestimable information in diagnosing and treating patients. Diagnostic qPCR usually relies on probe-based quantification for its enhanced specificity, and can be single-plex or multiplex, depending on the desired diagnostic information. A reference curve should always be used for diagnostic PCR, along with appropriate negative controls, regardless of whether absolute abundance is going to be reported. Multiplex qPCR is particularly relevant for diagnostic applications, as it allows the simultaneous testing for multiple pathogens in parallel, or the use of multiple probes targeting different sequences of the same pathogen, thus ensuring increased specificity of the test. Published reports suggest that ten or more pathogens can be screened for in a single multiplex reaction, making this qPCR application incredibly powerful[13]. In setting up a diagnostic qPCR experiment follow the recommendations in section 3.4, Multiplex qPCR design.

Additionally, care should be taken to establish a diagnostically relevant threshold for identifying samples as positive or negative for the target. This threshold should be clinically validated when qPCR is going to be used as a diagnostic tool. This includes estab-

lishing the specificity of the qPCR for the selected target, both in terms of the primers used to detect the target and in terms of target's suitability to accurately detect a potential pathogen and not similar targets in non-pathogenic strains. Furthermore, the limits of detection (LOD) and quantification (LOQ) should be established in accordance with relative peer-reviewed literature guidelines. Relative or quantitative accuracy of the qPCR test should also be estimated, and precision of the method must also be established. Refer to relevant scientific literature to find out more details on how these test parameters are established.

7.5 Genotyping

Another application of probe-based qPCR is allelic discrimination for genotyping. This specific use of probe-based detection allows the identification of different genotypes using probes with spectrally distinct fluorophores to target specific alleles in a multiplex reaction. The resulting fluorescent signal output allows the identification of which alleles are present in a specific sample. This approach is generally not quantitative (although such information can also be extracted from the same qPCR experiment) in that the amount of each allele is not calculated, but rather each probe's signal intensity is registered as present or absent when it's above a certain threshold value. The result is thus qualitative and binary (present/absent) for each allele. Combining the data on each allele for a single sample allows the genotype to be established (Figure 10).

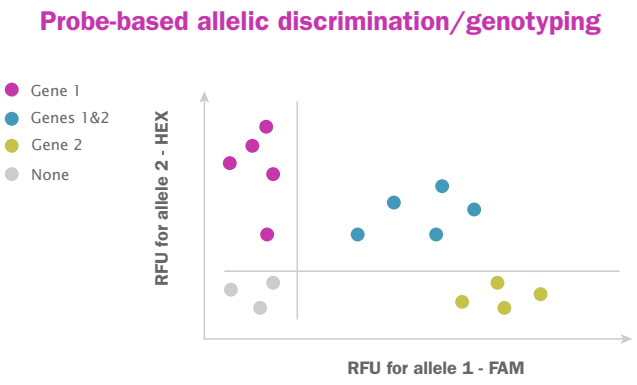


Figure 10

Allelic discrimination using probe-based qPCR

For example, in a simple bi-allelic state for a single locus with alleles A and B, two probes are designed, one specific to allele A and one to allele B and each respectively tagged with distinct fluorophores. These will generate a unique probe signal for individuals that are homozygous for allele A, a distinct unique probe signal for individuals that are homozygous for allele B and mixed signals from both probes from heterozygous individuals carrying both alleles A and B.

7.6 High Resolution Melt (HRM) curve analysis

HRM curve analysis utilises the power of intercalating dye-based qPCR in running a melt-curve analysis, combined with improved thermocycling instruments with finer control over temperature change increments. In a standard melt curve run after a qPCR, fluorescent data is acquired every 0.2-0.5 ˚C, whereas in HRM analysis data is acquired at a much higher density, every 0.1 ˚C, allowing for a much greater resolution in the resulting melt curve. This allows even small differences in sequence, down to single point mutations (SNPs), to register as shifted melt curves. HRM thus offers a cheaper effective alternative to probe-based detection for SNP detection, allelic discrimination or target variant detection. The compromise with this approach is reduced specificity of the method because a shift in melt-curves could also be the result of qPCR artefacts, as discussed in the limitations of Melt curve analysis (2.2). Care should be taken to validate results acquired by HRM analysis (Figure 11).

Figure 11

HRM for SNP and allelic discrimination studies

8. Guidelines for testing qPCRBIO products

We recommend you carry out these tests whenever you use one of our qPCR products or product samples for the first time. Following these recommendations will ensure the validity of your qPCR data.

8.1 General comments

Our qPCR mixes are available in a total of eight variants. Two basic mixes, regular and blue mix, each one of which is available with Lo-ROX, Hi-ROX, No-ROX or fluorescein (Table 4). All these mixes are equivalent in terms of performance and should be chosen in accordance with the passive reference dye requirements of the qPCR instrument to be used. Our probe mixes also come with all different types of ROX and are available with or without blue dye (Table 5). If you are uncertain of which mix is appropriate for your instrument, use our qPCR Selection Tool to find compatible mixes or contact technical@pcrbio.com with any further questions. No differentiation is made among these mixes regarding the guidelines recommended below.

Table 4: Passive dye option in qPCRBIO mixes.

qPCRBIO SyGreen Mix	qPCRBIO SyGreen Mix Blue
Lo-ROX	Lo-ROX
Hi-ROX	Hi-ROX
No-ROX	No-ROX
Separate-ROX	Separate-ROX
Fluorescein	Fluorescein

qPCRBIO Probe Mix	qPCRBIO Probe Blue MIx
Lo-ROX	Lo-ROX
Hi-ROX	Hi-ROX
No-ROX	No-ROX
Separate-ROX	Separate-ROX

8.2 Setting up test experiments

When setting up an experiment or comparing one of our mixes to competitor products, all primers, probes, template, diluent, and any other additives used to prepare reactions should originate from the same batch and preferably bottle. All reactions should be prepared on the same day, ideally by the same person. The same instrument and, if available, a liquid handling robot must be used for all comparisons.

Where absolute sensitivity is to be compared between the mixes, genomic DNA should be used. However, care must be taken not to over-interpret the results, because at low template dilutions stochastic effects take over. How to deal with this scenario is discussed in section 9, Troubleshooting.

Test experiments should be set up as follows:

- Test samples should include 4-5 template dilutions and a no template control (NTC).
- If reverse transcription reactions are carried out independently of the qPCR, i.e., you are not using a 1-step qPCR product, you should also include a no reverse transcription control that contains a true sample and all the same reagents as a regular sample minus reverse transcriptase.
- You should also use 3-4 technical replicates for each test and control sample.
- Template dilutions should be selected such that they cover a broad range, and all can contribute to a standard curve (a standard curve should at the very least consist of four points).
- Each reaction volume should be proportional to the support used (e.g., at least 20 µL for a 96-well plate) and the plate/tubes should be kept at 4 ˚C throughout the setup. Ideally, mastermixes as well (though these can be set up at room temperature since all polymerases are hot start).

Table 5 summarises the recommended test and control samples you should use when testing our products.

Table 5: Recommended experimental setup for qPCR product testing

Sample	Dilution 1	Dilution 2	Dilution 3	Dilution 4	No template control (NTC)	*No Reverse transcriptase control (NRT)
Content	*e.g.*, 10^{0} undiluted reference sample	*e.g.*, 10^{-2} dilution of Dilution 1	*e.g.*, 10^{-4} dilution of Dilution 1	*e.g.*, 10^{-8} dilution of Dilution 1	Sterile water as sample	Contains all reagents and sample but not reverse transcriptase
Replicates	x3-4	x3-4	x3-4	x3-4	Dilution x3-4 1	x3-4

*Necessary if you will be carrying out a separate cDNA synthesis step independently of qPCR, therefore it does not apply to qPCRBIO 1-Step mixes.

Care must be taken to avoid any type of contamination, particularly with samples that contain the target template at high concentrations, as these are often reported to contaminate NTC reactions. Use a clean bench or dedicated qPCR space for plate setup. The PCR plate must be sealed properly, *i.e.*, ensure full sealing of each individual well, and spin down in a microplate centrifuge to remove all air bubbles.

8.3 Cycling

Before running the comparison, cycling conditions must be optimised following each manufacturer's guidelines, to ensure that every mix performs at the best of its abilities for a given template-primer set.

If the optimised cycling conditions differ between the mixes, it is advisable to run each mix at its optimal conditions as well as each of the other competitors' conditions.

The same number of cycles must be used in the case of separate runs.

Each run should be followed by an identical melt analysis.

The plate should be examined after each run to make sure that it was sealed properly and that there was no reaction mix evaporation.

8.4 Data analysis

All wells showing spurious signal, or compromised reaction specificity, should be excluded from the analysis.

Differences in fluorescence intensity levels among different supplier's mixes are of little importance, provided they are within the same order of magnitude, unless a good rationale for such differences exists. The amount of dynamic range inherent in qPCR mixes is more than sufficient to explain such differences.

Direct comparison of different mixes poses a problem for threshold C_q estimation methods. Due to different fluorescence levels of different mixes, the threshold method can underestimate C_q values for high fluorescence level mixes. This can be mitigated by normalisation of the signal (essentially matching all plateaus). Please compare the raw signal to the output to determine whether your software performs such normalisation by default. If your software allows for C_q estimation by extracting the C_q parameter from curve fitting or if you can export the data and perform a custom-made analysis, then this will fix false differences between C_q values that arise from differences in fluorescence level. Otherwise, C_q values can be assigned by visualisation and moving the C_q threshold where possible with some software (*e.g.*, LinRegPCR)[10].

A standard curve should be calculated for each dilution series. Slope (efficiency) as well as intercept of the curve provide information on the quality of the PCR amplification. Efficiency of 2 (or 100%) is ideal, meaning that the amount of DNA doubled between each cycle. Efficiencies higher than that likely indicate error in the setup or the presence of inhibitors in the samples. Lower efficiencies suggest that the reaction conditions are not optimal: incorrect buffer, bad primer design, the wrong amount of dNTPs, Mg^{2+} or enzyme, or one of the components are degraded. Intercept provides a measure of the sensitivity of the assay. A lower value corresponds to higher sensitivity. However, this is a relative measure that

must only be used when comparing the same reaction setup (same amplicon, same cycling conditions, same instrument). Please note that we do not advise comparing C_q values generated with different supplier mixes at a single template dilution since the reactions may perform at different efficiencies.

The melt curves should be analysed for the presence of primer-dimers. The presence of a melt peak at a temperature lower than that of the PCR product indicates the formation of primer dimers. This is particularly visible at lower temperature dilutions and indicates poor hot start activity. Multiple peaks likely result from poorly designed primers or non-specific priming from inefficient hot start approaches. Peaks higher than that of the PCR product could indicate genomic contamination. The absolute position of the peaks often varies between different mixes manufacturers due to variations in buffer composition and should not be a source of concern.

9. Troubleshooting

Commonly occurring problems with qPCR are listed in the table below. There are other possible sources of error having to do with individual experimental conditions and obvious handling errors (e.g., failing to add reagents, or improper sample and reagent handling and storage) that are not included below.

Generally, good experimental design, primer and probe validation, careful sample preparation, and use of the proper positive and negative controls will help prevent issues and point to any sources of error. If facing persistent issues that are unrelated to your instrument's performance, contact your reagent supplier for further assistance. For PCR Biosystems reagents contact technical@pcrbio.com

9.1 Issues with dye-based qPCR

Problem	Cause	Solution
Target failure (no amplification) in experimental samples that should contain target, with correct amplification in positive control samples.	No or very low abundance of target present in samples	Repeat extraction if additional samples available. Concentrating samples by evaporation and resuspend. Ethanol precipitation and resuspension is also an option but increases sample loss.
	Inefficient cDNA synthesis	Repeat cDNA synthesis using appropriate amounts of good quality input RNA and following cDNA synthesis manufacturers guidelines. Try using a different combination of reverse transcription primers, including target-specific primers. If RNA quality is suspect repeat RNA extraction and verify RNA integrity.
	Pipetting error during master mix or template input	Repeat the experiment making sure template and all the other components are correctly added to every reaction well.
Target failure (no amplification) in samples that should contain target, including positive control samples.	Poorly designed primers	Re-design primers, ensure you follow all primer design guidelines.
	Primer annealing conditions are too strict	Reduce the selected annealing temperature and if not already done during primer validation run a temperature gradient to establish the optimal T_m for primer/probe sets.
	Error in setting up master mix, or in template input	Repeat the experiment making sure template and all the other components are correctly added to all reaction wells.
	Problem with the qPCR enzyme mix	Include or separately run reactions on a positive control sample to ensure all reagents are functioning properly. Try a different aliquot or order a new batch of qPCR mix.
Product in no template control	Pipetting error, incorrect template input during plate setup	Repeat the experiment making sure template and all the other components are correctly added to every reaction well.
	Contaminated mastermix or reagents	Repeat the experiment using a fresh aliquot of qPCR enzyme mix and primer dilutions.
	Contaminated work surfaces	Clean your work surface, use a dedicated PCR setup area in your lab, or use a clean bench.
	Contaminated pipettes	Disassemble and clean pipettes, autoclave pipettes (check with manufacturer that your pipettes are autoclavable). Use filter tips for qPCR plate setup, primer and sample dilutions, and mastermix preparation.

Problem	Cause	Solution
Low fluorescence in samples and positive control	Inhibitors present in the master mix resulting in reduced amplification and final yields	Re-purify samples, and check absorbance (A_{260}/A_{280}, A_{260}/A_{230}) to ensure purity.
	Reagent photobleaching, or repeated freeze thaw cycles	Ensure the reagent is within expiry day, avoid direct sun light, aliquot mix on arrival in volumes that will be used entirely in one preparation.
Linear amplification plot that rises above the threshold but remains linear until the end of the reaction.	Indicates reduced efficiency or inhibition of the qPCR.	Repeat the reaction making sure of accurate pipetting. Try further purification of the sample using phenol/chloroform extraction and ethanol precipitation or column-based purification. Some loss of nucleic acid is expected. If problem persists, re-extract nucleic acid from the sample or replace the sample with a comparable one.
Sigmoidal amplification plot that plateaus at much lower fluorescence levels compared to other samples	Fluorescence is being quenched by a component in the reaction.	See previous row for solutions.
	Too much input template.	Use less template.
High variation among technical replicates of the same biological sample	Low target abundance causing stochastic initial amplification.	Check sample nucleic acid concentration and integrity Use a larger amount of sample per reaction Repeat template extraction achieve a higher nucleic acid concentration Concentrate samples by evaporation (speed vac) or ethanol precipitation and resuspension in a smaller volume.
	Too much input template.	Use less template.
High variation among technical replicates of the same biological sample High variation among biological replicates within the same experimental treatment	Pipetting error	Repeat the experiment taking care to ensure problematic samples are properly set up If available, use a liquid handling robot
	Non-homogeneous sample due to presence of particulate matter (residual tissue, improperly dissolved reagents, contaminants)	Repeat extraction or clean up samples by centrifugation or ethanol precipitation using properly prepared solutions
	Could reflect natural variation in template amount	Run a statistical power test during experimental design, to ensure you have sufficient replication to overcome such variation.
High variation among biological replicates within the same experimental treatment Delayed amplification, late Cqs	High variation in template preparation, differing extraction/purification yields or efficiency of cDNA synthesis	Use similar amounts of starting sample (tissue mass, cell number, volume of biological fluid, *etc.*) for nucleic acid purification. Ensure thorough sample homogenisation during extraction. Poorly homogenised samples reduce final nucleic acid yield. If using cDNA as template, ensure reverse transcription of all samples is efficient, see relevant troubleshooting table.
	High variation in input template concentration	Ensure all biological samples are diluted to a similar total template concentration, ideally close to the midpoint of your reaction's linear dynamic range. Use similar amounts of template for all biological samples.
	Low target abundance	Verify input nucleic acid is within the recommended range for your application. Follow steps recommended in row 1 to increase target abundance.

Problem	Cause	Solution
Delayed amplification, late C_qs Shifted melt curve	Inefficient reaction conditions	Validate your experimental settings using positive control samples, try varying primer concentrations and reaction conditions. Redesign primers. Choose alternative target regions, if possible, e.g., reduce amplicon length to 80-100bp.
	Inefficient cDNA synthesis	See above
	SNPs in target significantly affecting annealing across samples	Verify the correct product being amplified by electrophoresis, or sequencing. If SNP variants should not be included in analysis redesign primers/probes to distinguish among variants.
Shifted melt curve Leading or double peak in melt curve	Non-specific amplification	Repeat sample purification to ensure no contamination. Verify primer/probe sequence specificity. Redesign primers/probes to different target regions.
	Contaminants in sample alter annealing temperature	Run an agarose gel to verify correct amplicon length. If in doubt run a restriction digest or sequence the product. If validated as the same product with shifted melt curve continue with analysis.
	Multiple products present	Increase reaction stringency, increase annealing temperature, decrease annealing time. Decrease total number of cycles. Verify primer specificity in silico and if necessary, redesign primers.
Leading or double peak in melt curve	Primer dimers or hairpins present (Early peaks with lower intensity or leading peaks often indicate primer secondary structure.)	Increase annealing temperature. If not already done, run a temperature gradient to identify optimal annealing conditions. Decrease primer concentration. Redesign primers.
	Product with bi-phasic denaturation (e.g., mammalian CFTR gene)	Verify single product by gel electrophoresis and sequencing. If correct product, ignore double peak and proceed with analysis as normal.

9.2 Issues with probe-based qPCR

Table 6: Troubleshooting common problems, causes and recommended solutions

Problem	Cause	Solution
Target failure (no amplification) in samples that should contain target, with expected amplification in positive control samples.	No or very low abundance of target present in samples	Repeat extraction if additional samples available. Concentrating samples by evaporation and resuspend. Ethanol precipitation and resuspension is also an option but increases sample loss.
	Inefficient cDNA synthesis	Repeat cDNA synthesis using appropriate amounts of good quality input RNA and following cDNA synthesis manufacturers guidelines. Try using a different combination of reverse transcription primers, including target-specific primers. If RNA quality is suspect repeat RNA extraction and verify RNA integrity.
	Pipetting error during master mix or template input	Repeat the experiment making sure template and all the other components are correctly added to every reaction well.
Target failure (no amplification) in samples that should contain target, including positive control samples.	Poorly designed primers and probe	Re-design primers and probes, ensure you follow all design guidelines.
	Primer annealing conditions are too strict	Reduce the selected annealing temperature and if not already done during primer validation run a temperature gradient to establish the optimal T_m for primer/probe sets.
	Error in setting up master mix, or in template input	Repeat the experiment making sure template and all the other components are correctly added to all reaction wells.
	Problem with the qPCR enzyme mix	Include or separately run reactions on a positive control sample to ensure all reagents are functioning properly. Try a different aliquot or order a new batch of qPCR mix.
Product in no template control	Pipetting error, incorrect template input during plate setup	Repeat the experiment making sure template and all the other components are correctly added to every reaction well.
	Contaminated mastermix or reagents	Repeat the experiment using a fresh aliquot of qPCR enzyme mix and primer dilutions.
	Contaminated work surfaces	Clean your work surface, use a dedicated PCR setup area in your lab, or use a clean bench.
	Contaminated pipettes	Disassemble and clean pipettes, autoclave pipettes (check with manufacturer that your pipettes are autoclavable). Use filter tips for qPCR plate setup, primer and sample dilutions, and mastermix preparation.
Low fluorescence in samples and positive control	Inhibitors present in the master mix resulting in reduced amplification and final yields	Re-purify samples, and check absorbance (A_{260}/A_{280}, A_{260}/A_{230}) to ensure purity.
	Photo-bleached fluorophore on probe due to improper storage	Use a fresh dilution of probe, or order a new batch if the issue is with the probe stock solution.

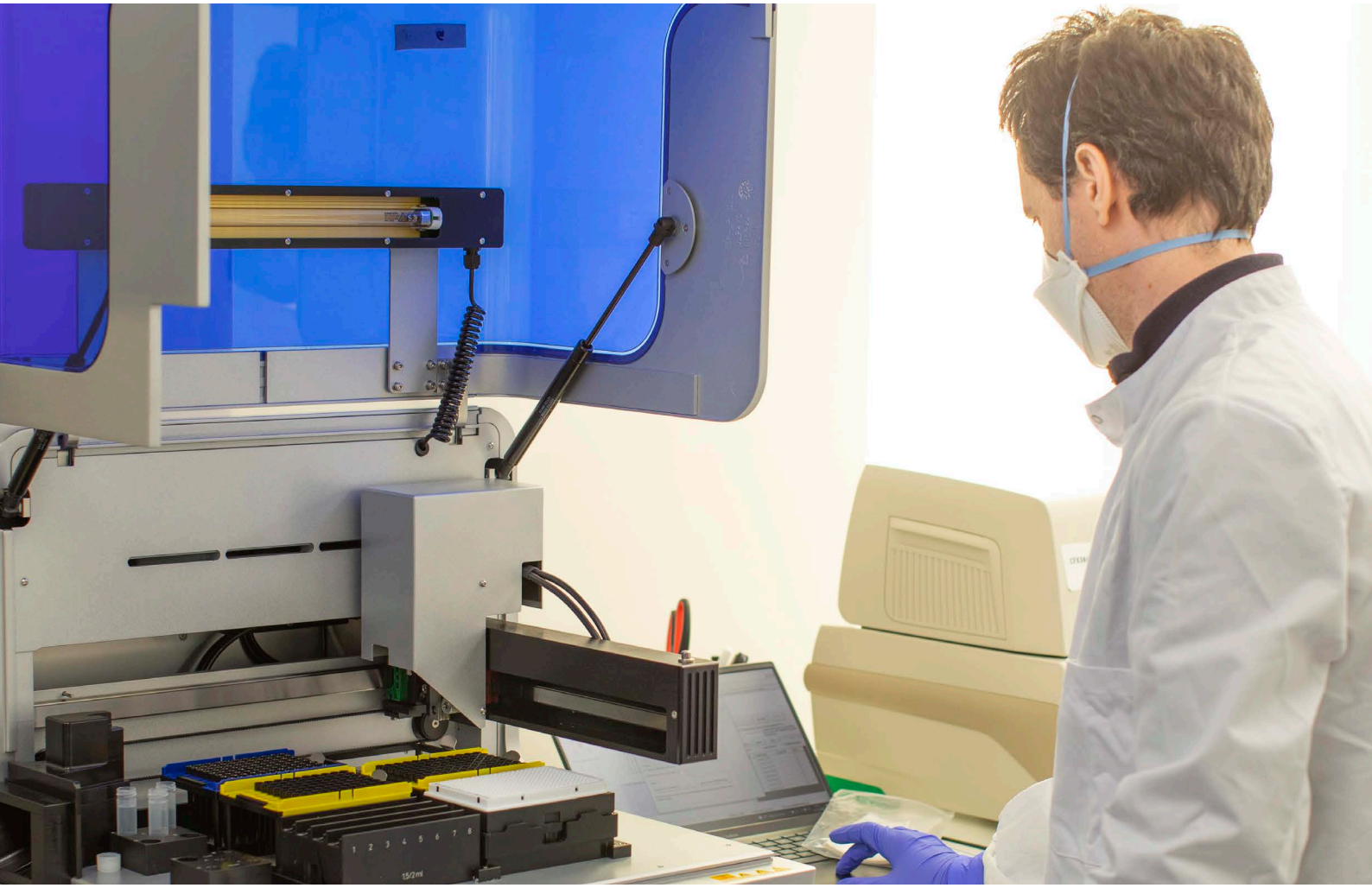
Problem	Cause	Solution
High background fluorescence	Degradation of the probe	Check NTC for possible increase in fluorescence in the absence of target. Use a new batch of probe
	Quencher noise	Some quenchers (*e.g.*, TAMRA) may also fluoresce. Ideally use dark quenchers and add internal quenchers.
	High concentration of input DNA	Reduce the amount of template, primer and probe input to minimum amounts that still allow efficient amplification.
	Improper handling of plate (e.g., air bubbles, fingerprint smudges, dirty plates/strip-caps/ sealing film)	Ensure no air-bubbles present in wells by plate centrifugation. Do not touch strip caps or sealing sticker without gloves, ideally hold plate from the edges not top or bottom of wells.
Linear amplification plot that rises above the threshold but remains linear until the end of the reaction.	Indicates reduced efficiency or inhibition of the qPCR.	Repeat the reaction making sure of accurate pipetting. Try further purification of the sample using phenol/chloroform extraction and ethanol precipitation or column-based purification. Some loss of nucleic acid is expected. If problem persists, re-extract nucleic acid from the sample or replace the sample with a comparable one.
Sigmoidal amplification plot that plateaus at much lower fluorescence levels compared to other samples	Fluorescence is being quenched by a component in the reaction.	See previous row for solutions.
High variation among technical replicates of the same biological sample	Low target abundance causing stochastic initial amplification	Check sample nucleic acid concentration and integrity Use a larger amount of sample per reaction Repeat template extraction achieve a higher nucleic acid concentration Concentrate samples by evaporation (speed vac) or ethanol precipitation and resuspension in a smaller volume
	Pipetting error	Repeat the experiment taking care to ensure problematic samples are properly set up If available, use a liquid handling robot
	Non-homogeneous sample due to presence of particulate matter (residual tissue, improperly dissolved reagents, contaminants)	Repeat extraction or clean up samples by centrifugation or ethanol precipitation using properly prepared solutions
High variation among biological replicates within the same experimental treatment	Could reflect natural variation in template amount	Run a statistical power test during experimental design, to ensure you have sufficient replication to overcome such variation.
	High variation in template preparation, differing extraction/purification yields or efficiency of cDNA synthesis	Use similar amounts of starting sample (tissue mass, cell number, volume of biological fluid, etc.) for nucleic acid purification. Ensure thorough sample homogenisation during extraction. Poorly homogenized samples reduce final nucleic acid yield. If using cDNA as template, ensure reverse transcription of all samples is efficient, see relevant troubleshooting table.
	High variation in input template concentration	Ensure all biological samples are diluted to a similar total template concentration, ideally close to the midpoint of your reaction's linear dynamic range. Use similar amounts of template for all biological samples.

Problem	Cause	Solution
Delayed amplification, late C_qs	Low target abundance	Verify input nucleic acid is within the recommended range for your application. Follow steps recommended in row 1 to increase target abundance.
	Primer and or probe secondary structures compete with template for primer/probe binding sites	Reduce primer and probe concentration. Increase annealing temperature to maximum possible without compromising reaction efficiency. Redesign primers and probes.
	Inefficient reaction conditions	Validate your experimental settings using positive control samples, try varying primer concentrations and reaction conditions. Redesign primers and probe. Choose alternative target regions, if possible, *e.g.*, reduce amplicon length to 80-100bp.
	Inefficient cDNA synthesis	See above

9.3 Issues with reverse transcription

Problem	Cause	Solution
Low yield	Low input RNA	Ensure you're using at least the minimum amount of RNA recommended by the RTase manufacturer Increase the amount of input RNA to the maximum recommended amount If RNA preparation is dilute, consider repeating RNA extraction or concentrating your sample by evaporation or ethanol precipitation and resuspension.
	Too much input RNA	Reduce the amount of input RNA to the minimum amount recommended by the RTase manufacturer. If necessary, dilute samples to achieve lower RNA concentration.
	Poor quality RNA template	Ensure RNA is of good quality, run on agarose gel or check RNA integrity on a Bioanalyzer. If necessary, repeat purification again making sure to check integrity.
	Residual DNase activity in RNA preparation	Check DNase used is heat labile. Ensure thorough inactivation of DNase after treating RNA during purification.
Contaminating DNA	Non-discriminating purification methods	Some traditional solvent-based methods do not discriminate well between RNA and DNA, leading to DNA enriched RNA preparations. Use appropriate salts, e.g., LiCl, for selective precipitation. If using TRIzolTM-type reagents, ensure you're not carrying over the DNA containing mid-phase during the organic solvent extraction step. Consider switching to column-based RNA purification kits. If using a column-based RNA preparation kit, opt for post elution DNase treatment instead of on-column digestion, as the latter is often inefficient. Regardless of purification method always perform a DNase treatment step and validate it's success by PCR.
	Failed DNase treatment	If using a column-based RNA preparation kit, opt for post elution DNase treatment instead of on-column digestion, as the latter is often inefficient. Ensure you do not exceed the maximum recommended amount of RNA in the DNase reaction. This amount is specified by most DNase manufacturers. Doublecheck DNase is active or use a new aliquot and perform longer DNase treatment.
	Didn't do DNase treatment	Regardless of purification method always perform a DNase treatment step and validate it's success by PCR.
	Contaminated equipment	Ensure your equipment and consumables, particularly pipette tips and sample tubes are nucleic acid free. Clean your working area and use a dedicated workspace for PCR-related procedures.
Excess of short cDNA fragments	Poor quality RNA template	Ensure RNA is of good quality, run on agarose gel or check RNA integrity on a Bioanalyzer. If necessary, repeat purification again making sure to check integrity.
	Used only oligo-dT or only random hexamer primers	Use a mix of random hexamers and oligo-dT primers.
	Short incubation time	Increase the incubation time to the maximum recommended by your RTase manufacturer. Most protocols opt for a faster extension time that is suitable for most applications, but this may reduce first strand cDNA length.

Problem	Cause	Solution
3'-enriched cDNA, underrepresented 5' ends	Used only oligo-dT primers	Use a mix of random hexamers and oligo-dT primers in order to ensure you get good coverage of full-length transcripts.
	Short incubation time	Increase the incubation time to the maximum recommended by your RTase manufacturer. Most protocols opt for a faster extension time that is suitable for most applications, but this may reduce first strand cDNA length.
Failure to detect expected transcripts	Low input RNA	Ensure you're using at least the minimum amount of RNA recommended by the RTase manufacturer. Increase the amount of input RNA to the maximum recommended amount.
	Poor quality RNA	Ensure RNA is of good quality, run on agarose gel or check RNA integrity on a Bioanalyzer. If necessary, repeat purification again making sure to check integrity.
	Inefficient priming	Ensure thorough template denaturation, by pre-incubating template and primers at 65-70 °C degrees for 5min and then rapidly cooling down on ice before starting the reverse transcription reaction. If possible (e.g., for transcript cloning or 1-step RT-qPCR), use a gene specific primers for first strand cDNA synthesis, instead of generic primers (random hexamers and oligo(dT).



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11. Disclaimer

Disclaimer

This manuscript has not undergone peer review.

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