Overcoming the challenges of qPCR assay design

Expert tips and guidance

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GENOMIC ANALYSIS BY LGC

Gain the competitive edge

Whether you are working in an emerging or established lab, launching a quantitative PCR (qPCR) experiment requires a deep understanding of complex principles, protocols and methodologies. Parsing through the massive amounts of published qPCR data to select the best experimental framework for your objectives requires a significant amount of time, expertise and resources.

This guide helps qPCR assay developers to get their experiments underway by providing concise summaries of experimental design considerations within the assay development process, and applying established guidance to overcome challenges at every stage. You will learn how to apply the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009) to produce top-quality assays for molecular diagnostics. By adhering closely to the MIQE guidelines, you will be able to identify issues within your qPCR assays through a step-by-step troubleshooting approach.

At LGC Biosearch Technologies, we harness the power of bioinformatics, leading-edge assay design tools and decades of experience to design the best qPCR assay or genotyping assay for your target and application. To get proactive, hands-on support services from technical experts, contact our PCR assay design and development team or visit this page to learn more and request bespoke tech support.

Contents

Abbreviations

Abbreviations	Definition
C_q	quantification cycle
EQC	external quality control
IQC	internal quality control
LOD	limit of detection
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
NTC	No template control
QC	quality control
qPCR	quantitative polymerase chain reaction
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SNPs	single nucleotide polymorphisms
T_m	melting temperature

qPCR: Techniques and best practices

The enduring legacy of qPCR

For the better part of the last 40 years, fluorescent probe-based qPCR has maintained its reputation as the most popular technique for detecting and quantifying low levels of target nucleic acids from many sample types with exceptional specificity (VanGuilder et al., 2008).

Researchers use qPCR to monitor how much of the target amplicon is present at each amplification cycle, enabling the quantification of the initial amount of the target in each sample. Beyond specificity, benefits of qPCR include an extended detection range compared to conventional PCR methods and a streamlined workflow that combines amplification and detection steps, reducing the risk of contamination or sample loss.

The need for guidelines

As qPCR garnered favour across many applications for its speed, sensitivity and specificity, a variety of methods emerged for every step of the process. The many flavours of qPCR assay design make the technique flexible and adaptable. However, this added complexity can make it challenging to design an assay that meets researchers' aims with accurate, reproducible results.

Recognising that consistent methods and transparent reporting would boost the quality and value of qPCR assays, the MIQE guidelines were published to give researchers a set of trusted recommendations for developing qPCR experiments (Bustin et al., 2009). The aim of the MIQE guidelines is to:

- Help researchers design, report and publish better and more robust qPCR studies
- Provide authors and reviewers with a set of established criteria
- Improve reproducibility of future experiments

Read on to learn how to apply these guidelines to design an effective qPCR assay for your experimental conditions.

Control considerations

Maintain the integrity of your qPCR assay by including a set of quality controls (QCs) in the design. Controls are used to monitor reaction conditions, performance, contamination and background noise. Validating your experimental conditions helps ensure that your assay is functioning correctly and generating accurate results.

Types of controls

No template control (NTC)	External quality control (EQC)	Internal quality control (IQC)
NTC samples contain all the PCR components except the target nucleic acid sequence. These are used to monitor for contamination that may produce a false positive result and to detect primer dimer products.	EQC samples have a known result and are run alongside the test samples. These are typically included at each stage of the workflow (extraction/purification, reverse transcription, PCR). EQCs include both positive and negative controls and are used to verify experimental accuracy and control for contamination events.	IQC samples are created by spiking additional material of a known concentration into the test samples. These IQCs are tested in parallel with the sample to control for inhibition events and discern between a true negative and a false negative.

Our scientists advise:

The unintended introduction of nucleic acids or PCR products into samples can cause inaccurate and unreliable results. High copy number amplicons and non-specific amplicons can easily transfer between lab equipment and build up in lab spaces. Therefore, take measures to avoid exposing template material to pre-PCR set-up spaces and reaction components.

Prevention is the best defence against contamination. Keep pre- and post-PCR areas separate and implement decontamination protocols when moving between the two. We recommend using a 'one-way system' as an optimal method to minimise the chances of contamination.

Please note that non-specific hybridisation is a genuine experimental result but may appear as a contamination event in NTCs.

For reverse transcription qPCR (RT-qPCR), run a no-reverse transcription control to check that the assay is detecting cDNA rather than contamination from gDNA.

When possible, design gene expression assays to target exon junctions to prevent detection of genomic DNA.

See our qPCR contamination assessment and mitigation guide.

Sample considerations

A successful qPCR assay depends on choosing the optimal sample extraction, handling and processing methods for your goals. Maximise the accuracy and reliability of your results by following best practices across the recommendations listed below. Detailed reporting of each step facilitates appropriate data evaluation and comparison of results.

Our scientists advise:

Achieve maximum confidence in qualitative and quantitative results with thoughtful qPCR assay design and optimisation. Follow these recommendations to design the best qPCR assay for your target of interest and experimental conditions.

Recommendation 1: Sample purification

Final observed results are impacted by the quality and integrity of extracted nucleic acids.

Treat biological samples to ensure that extracted and purified nucleic acids (if applicable) meet minimum testing criteria as outlined in the Good practice guide for the application of quantitative PCR (qPCR), including concentration, purity and absence of inhibitors (Nolan et al., 2013). Note that co-purified contaminants may influence the final observed result.

Recommendation 2: Sample-to-sample standardisation

Variability in nucleic acid quality and degradation levels among samples can lead to inconsistencies within the same assay, impacting overall results.

Measure isolated nucleic acids to ensure minimum quality/quantity requirements are met using methods such as 260/280 and 260/230 spectrophotometric readings. Fluorometric measurements using double-stranded nucleic acid intercalating dyes can also be used.

Recommendation 3: Inhibition

Products or artefacts from sample processing can interfere with the amplification and detection of the target nucleic acid by downregulating or enhancing the observed result. This can lead to difficulty assigning genotypes or correctly interpreting relative target quantities.

Include an internal quality control with each sample to be tested to assess the presence of inhibition (see control considerations above).

See our qPCR contamination assessment and mitigation guide. **When should you reach out to the Biosearch Technologies assay design and development team?** If you have questions about sample extraction, processing, or how to implement appropriate controls for your assay, research objectives and lab capabilities.

Primer and probe selection

Design or select primers based on your priorities and research objectives. In addition to following general design guidelines for selecting melting temperature (T_m), GC content, optimal primer length, etc., it is important to consider these other aspects of the target sequence:

- Carefully review amplicon templates for secondary structures and repetitive regions to select primers and probes specific to your target of interest.
- Is your target evolving quickly? Consider multiple targets to build variation tolerance and enhance sensitivity of your assay.

Reverse transcription (RT) primer selection

- Make sure your RT primer does not target a region with single nucleotide polymorphisms (SNPs), which can destabilise binding and impact sensitivity.
- If running a multiplex RT-qPCR reaction, do not limit the RT primer to optimise the assay. See the Multiplex design section to learn about primer limiting optimisation strategies.

More guidance on probe selection: A how-to guide for qPCR probe selection e-book and our online probe selection tool.

Multiplex design

Using multiple sets of primers and probes for different targets within the same qPCR can expand your research capacity but requires additional assay design and optimisation considerations:

- Use *in-silico* tools to assess multiplex compatibility of your targets and help select reporter dyes to minimise crosstalk and reduce unnecessary trial and error during wet-lab assay optimisation.
- Select primers that are not predicted to form primer dimers, which reduce assay efficiency.
- Validate the performance of each assay as a singleplex before combining in a multiplex, to ensure each target is being detected accurately with acceptable sensitivity.
- Perform additional primer optimisation studies to assess performance when targets are combined. Adding more primers increases the risk of primer dimers, potentially diminishing sensitivity compared to singleplex reactions.
- Consider limiting the concentration of one primer, but not the RT primer. This hastens the reaction plateau for the control target, reserving enzyme, dNTPs and cations for test targets.
- Use a master mix which supports multiplex qPCR reactions. We recommend BHQ Probe Master Mix or RapiDxFire qPCR 5X Master Mix GF, both offering robust performance to drive operational efficiency of qPCR reactions.

When should you reach out to the Biosearch Technologies assay design and development team? To perform *in-silico* validation to assess and inform optimisation of primer and probe design and selection.

Assay optimisation

Once your assay is designed, optimisation is crucial to ensure robust performance. Optimisation requires testing primer concentration for the combination that delivers the best sensitivity. Major aspects of optimisation include:

- Selecting positive and negative controls to confirm the assay continues to function as expected
- Determining assay limit of detection (LOD)
- Avoiding making changes to components and conditions once the assay is optimised and validated
- Tracking any variation over time with reference standards

Furthermore, running replicates and randomising sample arrangement increases the confidence of your results, reduces sources of bias and prepares the data for statistical analysis.

Replicates

Operator handling and biological variability can affect the consistency and reliability of results.

Use replicates to account for variability and ensure reproducibility of results. There are two types of replicates in qPCR assays:
Biological replicates: Multiple biological samples either from different sources (e.g., different patients) or different sample types from the same source. These account for natural biological variability and are vital for statistical analysis.
Technical replicates: Multiple reactions using the same sample and PCR reagents performed in different tubes or wells.
The exact number of biological and technical replicates needed for your experiment will vary based on the required resolution and the reproducibility of your assay. A general rule of thumb is to start with 3 biological replicates and 2-3 technical replicates.

Randomisation

Differences in spatial or positional factors can introduce sources of bias and impact results.

Maximise the likelihood that any observed differences are due to true biological variation and not bias within the experimental set-up by incorporating randomisation into the assay design (e.g., randomly assigning samples and controls to different wells or positions on the PCR plate).

Our scientists advise:

We recommend ensuring your qPCR performs at optimal efficiency with the best possible sensitivity, specificity and precision by carrying out in-house optimisation and validation of each qPCR assay prior to routine use. To precisely characterise sensitivity, specificity and precision:

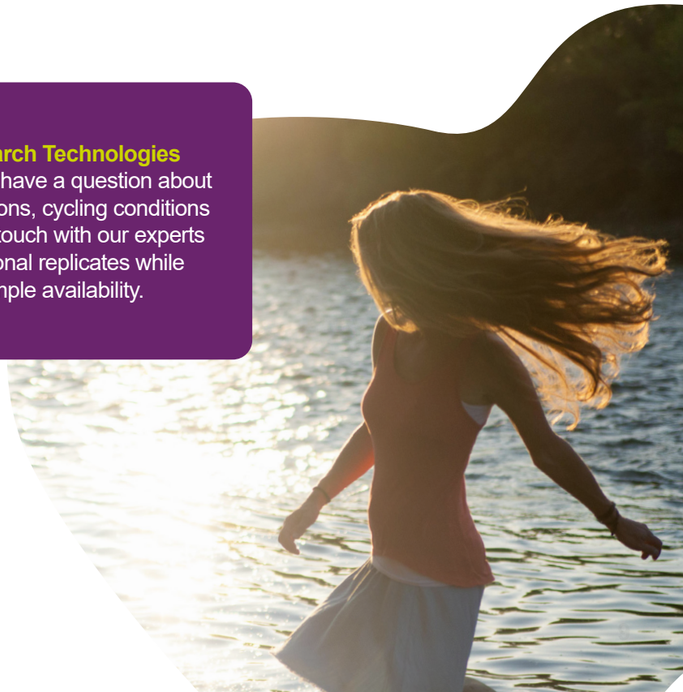
Optimise concentrations of primers and probes to the highest technical reproducibility at the lowest limit of detection, with any NTCs remaining a true negative.

Check that positive internal and external quality controls remain positive if you are working near the limit of detection. Typically, positive controls generate values with a high quantification cycle (C_q), which provides confidence in the ongoing robustness of the assay and minimises the risk of these controls contaminating adjacent samples.

Choose between a two-step or three-step thermal cycling protocol. A two-step cycle (a denaturing step and a combined annealing/extension step) reduces cycling time and accommodates qPCR assays with different target lengths, GC contents, amplification efficiencies and other properties, but limits the scope for oligonucleotide design and does not support melting temperature optimisation.

Decide on the best magnesium chloride ($MgCl_2$) concentration. $MgCl_2$ impacts hybridisation of the oligos to the target, the processivity of the DNA polymerase enzyme and the rate of hydrolysis of the exonuclease moiety. Too little $MgCl_2$ can detract from performance, but too much can cause non-specificity. qPCR applications may require as much as 3-5 mM $MgCl_2$ to achieve sufficient probe cleavage to generate a fluorescent signal. The concentration of $MgCl_2$ is usually fixed in a given buffer, so optimise your assay in the buffer you will use and re-optimise if changing buffers.

When should you reach out to the Biosearch Technologies assay design and development team? If you have a question about determining optimal oligonucleotide concentrations, cycling conditions and $MgCl_2$ concentrations. Consider getting in touch with our experts who can help you assess the need for additional replicates while taking into account costs, space and sample availability.



Assay validation

After designing and optimising your assay, it is important to assess the key performance parameters that can impact its efficiency, technical dynamic range and reproducibility. This crucial stage will inform next steps and future development potential, ultimately determining whether your qPCR assay meets the needs of your target research or diagnostic application.

Key performance parameters

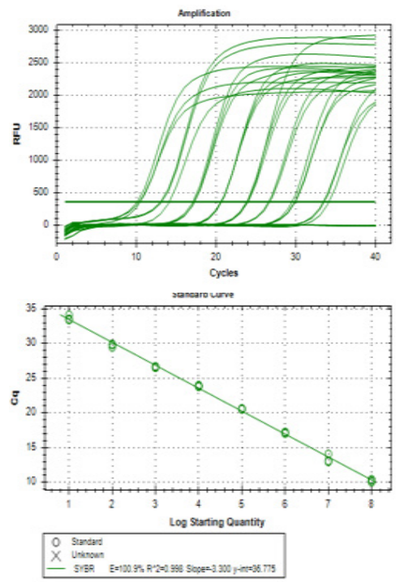
- **Precision** – The closeness of agreement between independent measurements.
- **Bias** – The difference between the expected test measurement and an accepted reference value.
- **Robustness** – Tolerance for potential experimental and/or operator errors, which could accumulate over time.
- **Specificity** – The extent to which the methods can detect the target without interference from other, similar components.
- **Sensitivity** – The reproducibility to identify the lowest, defined limits of detection.
- **Working range and linearity** – The interval between the upper and lower concentrations of the target, deemed suitable for the assay, and the assay's ability to generate a result directly proportional to the concentration of the target.
- **Measurement uncertainty** – The estimated range of values within which the true value of the measurement resides, indicating the reliability of the assay.

Standard curve

The most commonly used method to assess the performance of an assay is to generate a standard curve, which relates the cycle quantification (C_q) values of a known concentration of your template to their corresponding initial template concentrations.

Follow these steps to generate a standard curve:

1. Prepare ten-fold serial dilutions of known concentrations of your template in your desired detection range. Samples used to generate standard curves can be synthetic templates, plasmids containing the target of interest or cell culture extractions. At least five serial dilutions are needed to create a standard curve.
2. Perform qPCR for each of these known concentrations.
3. Measure the C_q value for each known concentration. The C_q value is the number of qPCR cycles required for the fluorescence signal to exceed background levels.
4. Plot the C_q values against the logarithm of the concentration to create a standard curve.



You can now use your standard curve to check the efficiency of your assay. The assay efficiency is calculated by the slope (m), derived from the line of best-fit, described by the equation $y = mx + c$ and where the efficiency is calculated as $E = 10^{(-1/m)} - 1$. Reaction efficiencies between 90% and 110% are typically acceptable, i.e. a slope between -3.10 and -3.58.

It is important to assess the linearity over the whole concentration range. The R^2 value indicates how well the data fits on the standard curve straight line and is a measure of reproducibility. You may need to tweak your assay if the standard curve has any of the following characteristics:

1. $R^2 \leq 0.985$: Your assay results may not be reliable overall.
2. Non-linearity at the highest concentration: Your reaction may be saturated and the target concentration is beyond its effective measurement range.
3. Non-linearity at low template concentrations: The concentration may exceed the sensitivity of your assay.
4. Non-linearity at random points: Pipetting accuracy is compromised or the assay may need to be re-optimised.

When should you reach out to the Biosearch Technologies assay design and development team?
For assistance in calculating and evaluating key performance parameters, generating a standard curve and assessing assay efficiency. For more information on standard curve troubleshooting, refer to this blog post.

Assay normalisation

Normalisation helps produce a true result by accounting for variations in extraction/purification yield, reverse-transcription yield, and amplification efficiency. Normalisation should account for possible technical variability in each step of the qPCR protocol.

Types of normalisation

	Biological sample normalisation	Assay normalisation	Analysis normalisation
⚠	Most biological samples are inherently heterogeneous with differences in cell count, nucleic acid concentration and composition.	Operators can introduce variation during sample preparation, processing or experimental protocols.	The total amount of fluorescence signal read can be affected by variation in the amount of reaction mix added to tubes, wells or in the optics shuttle light-path between wells.
✓	Normalise extracted/ purified nucleic acids by routinely measuring with absorbance-based or fluorescence-based methods.	Include internal (e.g. measure reference gene expression) and external quality controls. Controls with known concentrations/copy numbers enable assessment of factors associated with each sample that can affect the assay's PCR efficiency. See page 4 for more guidance regarding controls.	Include a passive reference dye like ROX in the reaction mix. This can be used to measure and normalise fluorescence values of the fluorophores associated with the target-specific amplification.

When should you reach out to the Biosearch Technologies assay design and development team?
To identify sources of potential variation, implement appropriate normalisation steps and select a quantification method for your assay.

Quantification methods

You are now ready to run your assay and use either standard curve or comparative quantification to determine the quantities of your targets in test samples.

Types of quantification

Standard curve quantification	Comparative quantification (delta delta C_q or $2^{-\Delta\Delta Cq}$)
Using the standard curve to determine the concentrations of unknown test samples. When using this method, keep the threshold setting constant for determining C_q for the standard curve and test samples on the same plate. The threshold can differ between plates.	• Measuring the relative change in gene expression levels under different experimental conditions or over time. • The concentration of the target gene is compared against a well-established reference gene helping to minimise the impact of any variations introduced by operator or experimental conditions. It is recommended that multiple reference genes are included (usually three) and analysed using geNorm • This method assumes both the target gene and the reference gene have identical amplification efficiencies close to 100%. If the amplification efficiencies differ significantly, this can lead to inaccurate results. In this case, consider using the Pfaffl method, which overcomes this limitation by taking into account differences in amplification efficiencies of both the target gene and reference gene (Pfaffl, 2001).

Data analysis

Data analysis in qPCR involves collecting and reviewing the raw data, assessing its quality and reliability, and generating results that can be reported. Researchers should report detailed information on their data analysis strategies and confidence, including software used, statistical methods and how accuracy was assessed (Bustin et al., 2009).

	Baseline correction	Setting a threshold
⚠	• Baseline fluorescence may affect amplification curves and hinder the quantitative comparison of different samples. • Some factors that can contribute to baseline fluorescence include: ✓ Choice of plasticware ✓ Unquenched probes ✓ Signal carryover into the neighbouring sample wells	The threshold should exceed baseline fluorescence and denote the exponential amplification phase. qPCR software may set the threshold incorrectly, affecting C_q values, potentially missing detection of samples with lower amounts of template or variations of C_q values between different samples and controls, thus affecting relative quantification.
✓	• Use fluorescence observed in the early stages of the qPCR run to identify the linear component e.g. cycles 3-10, and normalise the rest of the sample signals against these readings. It's important to pinpoint which cycles to use for baseline fluorescence, since more cycles increase the potential accuracy of the linear component but also result in increased fluorescence from target amplification, making these readings unsuitable for baseline correction. Please note it is essential that any cycle with increased fluorescence is not included in baseline normalisation. Be aware that some qPCR instruments have automatic settings that need to be adjusted to your specifications. • Use dye calibration standards to optimise signal detection and limit crosstalk between fluorescence channels.	Read the cycle from each log-linear curve to determine quantities for each sample. Compare samples on the same reaction run since the threshold is set at the same point for all samples tested. Set the threshold in the log region of amplification, above the fluorescence baseline so no amplification curves prematurely cross it, and as low as possible so that the threshold crosses the log-linear phase of each sample and not the plateau phase. Adjust the threshold for different dyes to account for differences in fluorescence.

When should you reach out to the Biosearch Technologies assay design and development team?
If you are uncertain how your available components affect baseline fluorescence, which cycles to use for baseline correction or how to set your threshold.

Getting the most out of your qPCR assay

Partner with the qPCR assay design and development scientists at Biosearch Technologies to get expert support.

Services include:

- **Expert selection** of primers and probes specific to your target of interest, assay type, and experimental goals
- **Flexible approach to chemistry selection** and access to an expansive selection of probe chemistries, including BHQ™, BHQplus™, BHQnova™, Minor Groove Binder (MGB) probes, Locked Nucleic Acid (LNA) probes, Scorpions Primers™ and Molecular Beacons
- **Multiplexing options** for your specific instrumentation
- **Rapid synthesis and re-design**, with one alternative assay design at no additional charge if needed
- **Fine-tuning variables** to optimise candidate design
- **Advice and support** with submitting your assay for manufacturing

Ready to get started?

Support services are available at affordable rates, starting as low as a few hundred dollars per assay, depending on the target density.

To begin, simply fill out this project request form with a few details about your objectives.

References

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