10 tips to maximise qPCR success

Need help getting reliable, consistent qPCR data? Here are a few pointers from the scientists at LGC, Biosearch Technologies™.

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Before you begin

The path to good data starts before you even pick up a pipette.

1. Review MIQE guidelines

Why? Produce publication-quality data

The Minimum Information for Publication of qPCR Experiments (MIQE) guidelines provide community standards designed to maximise the quality, reproducibility, and comparability of qPCR studies. Further information can also be found in the Good practice guide for the application of quantitative PCR (qPCR).

2. Aliquot and store reagents properly

Why? Maintain performance and reduce contamination risks

In addition to ensuring that all reagents are stored and handled as recommended by the manufacturer, aliquoting large-volume reagents into single-use volumes can limit the potential for contamination and also avoid freeze-thaw cycles that damage oligonucleotides and qPCR master mixes. When aliquoting, be sure to use nuclease-free tubes and, for light-sensitive reagents, make sure the tubes are light-protective.

3. Use Good Laboratory Practice (GLP)

Why? Ensure safety, promote efficiency, and reduce contamination risks.

- Wear appropriate personal protective equipment (PPE) such as laboratory coats and disposable gloves.
- Use nuclease-free, molecular biology-grade reagents and plasticware, including water, disposable pipette tips, tubes, and plates.
- Use filtered pipette tips.
- Clean equipment regularly according to manufacturers' instructions.

To further reduce contamination risks, designate separate work areas for steps:

- Without nucleic acid, such as setting up qPCR master mix.
- With nucleic acid, such as extraction, purification, and addition to the qPCR reaction.
- After the qPCR reaction.

Designing your study

Smart experimental design choices can boost the quality of your data.

4. Include sufficient replicates and controls

Why? Maximise data quality, interpretability

- Choose sufficient replicates to achieve sensitivity and specificity goals.
- Include both biological replicates (multiple independent samples) and technical replicates (the same sample used in multiple independent reactions).
- Use non-template controls (NTCs) – reactions without target DNA – to identify contaminating amplifiable DNA.

- Use external quality controls (EQC) – samples with a known result – for each step to verify performance, *i.e.* extraction/purification and qPCR reaction.
- Use internal quality controls (IQCs) – "spike-in" DNA – to distinguish true negatives from failed reactions.
- Randomise the location of controls and samples in your plate to reduce systematic biases introduced by pipetting, instrumentation, or the environment.

5. Optimise oligonucleotide design

Why? Reduce noise and ensure accurate quantification

- Perform BLAST alignments to minimise off-target binding.
- Check the oligonucleotide for self-complementarity to avoid secondary structures, primer-dimers, hairpin loops, and concatamers.
- Ensure that all oligonucleotides within the same reaction have a similar melting point.

Simplify oligonucleotide design with Biosearch Technologies' RealTimeDesign software.

6. Use robust fluorophores and quenchers

Why? Maximise signal-to-noise ratio

- Select a "true-dark" quencher (i.e. Black Hole Quencher™ (BHQ™) compatible with your filter channels.
- For multiplex qPCR, choose fluorophores with little-to-no spectral overlap.
- Select a "true-dark" quencher that has no fluorescence emission to minimise background signal.

Need help choosing? The online Spectral Overlay Tool facilitates selection of appropriate fluorophores and BHQ quenchers for your qPCR instrument.

7. Reduce pipetting by using working assay mixes

Why? Improve data consistency and accuracy, reduce contamination

- Making 20x, 40x, or 80x working mixes of all oligonucleotides and adding the working mix to the qPCR reaction ensures consistent oligonucleotide ratios across multiple reactions, reducing variability and limiting contamination risks and pipetting errors.

Get help making working mixes and oligo dilutions in the BHQ Probe Master Mix user guide with the Biosearch Technologies Oligo Toolbox.

Or use Biosearch Technologies' ValuMix for Gene Expression, which is a ready-to-use blend of primers and probes for qPCR applications.

Getting to work

Maximising sample integrity is critical for a successful outcome.

8. Use high-quality target nucleic acid

Why? Maximise polymerase activity, minimise reaction inhibitors

Assess nucleic acid quality using absorbance for general qPCR assays, or fluorescence for highly-sensitive qPCR assays.

Absorbance:

- $OD_{260/280}$ should be between 1.8 and 2.1 to avoid contaminating protein or phenolic compounds.
- $OD_{260/230}$ should be close to 2.0 to minimise organic solvents such as TRIzol or chaotropic salts.

Choose a nucleic acid extraction and purification method based on target species, genome size, qPCR parameters, cost, time, and downstream applications. Biosearch Technologies' sbeadex is a robust, magnetic-bead based purification system for easily-automatable isolation of high-quality nucleic acid. Cruder methods, such as the classic sodium-hydroxide based "HotShot" method, or Biosearch Technologies' QuickExtract Plant Extraction Solution, can offer fast, simple nucleic acid extraction for some sample types.

Analysing the data

The best data means nothing without rigorous attention to the quality of the analysis.

9. Use rigorous data handling practices and qPCR analysis

Why? Ensure data accuracy

- Reduce the impact of background fluorescence by using a baseline correction that maximises the distance between peak background signal and max signal.
- Set the threshold to intersect the exponential point of each amplification curve.
- Set individual threshold values for different assays, even if they are run on the same plate.
- Check whether you are in linear or logarithmic mode before setting the threshold value.

- For assays with non-sigmoidal amplification curves, check for inhibition of PCR efficiency using IQCs. If needed, remove any non-specific amplification curves.
- Apply any normalisation (via the passive reference dye) as required, to account for any differences due to pipetting discrepancies.

10. Assess the robustness of standard curves

Why? Increase confidence in quantification of unknown targets.

For each standard curve, consider performing the following calculations:

- Aim for a PCR efficiency of 90-110%. The PCR efficiency is the ratio of the number of target gene molecules at the end of a PCR cycle divided by the number of target gene molecules at the start of the same PCR cycle.

- Aim for an R^2 value close to 1. R^2 is the coefficient of variance between replicates at a single concentration.

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