Troubleshoot your qPCR

This guide is witten to help researchers rapidly troubleshoot errors that have occured in their qPCR experiments. For users new to qPCR we recommend refering to our full qPCR Technical Guide before beginning their experiments.

In the table below you can easily find problems with:

- Dye-based qPCR
- Probe-based qPCR
- Reverse transcription in RT-qPCR

In this table, commonly occuring errors are listed in the first column, probable causes in the second column, and the recommended solutions to each problem are found in the third.

Sources of error having to do with obvious mishandling (*e.g.*, the user failing to add reagents, or improper sample and reagent preparation and storage) 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 such issues and point to any sources of error.

If facing persistent issues that are unrelated to qPCR instrument malfunction, contact your reagent supplier for further assistance. For PCR Biosystems reagents contact technical@pcrbio.com.

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 target or very low abundance of target present in samples, particularly an issue with low-expression genes and or rare species in complex samples.	Repeat extraction if additional samples available. Concentrate 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 in 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 Tm 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.

Problem	Cause	Solution
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.
	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.

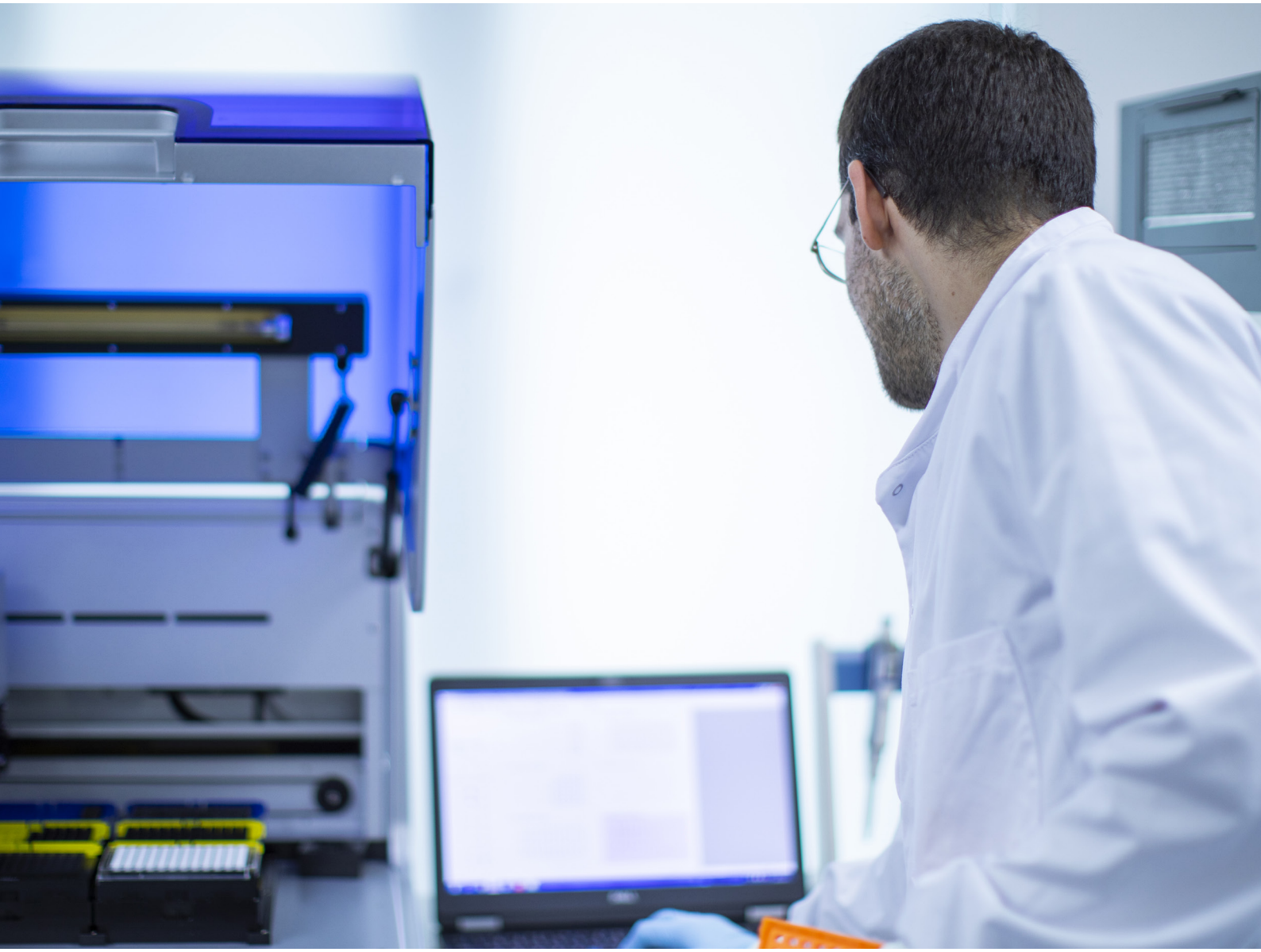
Problem	Cause	Solution
High variation among biological replicates within the same experimental treatment Delayed amplification, late Cqs	High variation in template preparation, differing extraction/ purification yields or efficiency of cDNA synthesis	Use similar amounts of starting sample (tissue mass, 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.
Delayed amplification, late Cqs 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 80-100 bp.
	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.

2. Issues with probe-based qPCR

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 Cqs	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 80-100 bp.
	Inefficient cDNA synthesis	See above



3. Issues with reverse transcription in RT-qPCR

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).