

Problem	Possible Causes	Actions
No amplification or very high Ct	Enzyme not fully activated	Ensure the initial 95°C activation step is carried out for the full 15 minutes.
	Poor primer design	Check the PCR product by melt curve analysis or on an agarose gel. It is good practice to try at least 2 primer pairs.
	RT step too short	Extend the RT step in 5 minute increments up to 60 minutes
	RT temperature too low	Increase RT reaction temperature in 5°C increments up to 57°C
	Annealing step too short	Increase annealing step in 3s increments up to 30s
	Annealing temperature too high	Decrease the annealing temperature in 2°C increments
	Extension time too short	Increase the extension time in 5s increments, up to 30s for amplicons of up to 500bp.
	Amplicon too long	Amplicons should ideally be 100-150bp long and should not exceed 500bp
	Poor template quality	Check the quality of the template preparation using spectrophotometry, microfluidics or PAGE.
	Insufficient template	Increase amount of template to ensure enough copies of target included
	Template contains inhibitors	Purify template or repeat the assay using a 1:10 or a 1:100 dilution of the template
	Insufficient cycles	Increase the number of PCR cycles to 40
	Wrong dye/channels used	Check machine settings correspond with dye from probes and ROX/fluorescein levels are correct.
	Error in setup	Check concentrations and storage conditions of the reaction components and repeat the reaction
	Reaction components not mixed thoroughly	Repeat assay ensuring serial dilutions are vortexed for at least 15s and that the reaction components are mixed together thoroughly
	Primers degraded	Check the integrity of the PCR primers by denaturing polyacrylamide gel electrophoresis
	Primer concentration not optimal	Start with primer concentration recommended in protocol and increase primer in 25mM increments.
	Fluorescent data collected at wrong step	Ensure fluorescent data is collected during the extension step and the correct channel is used
	Fluorescent reporter not being released from probe	Validate performance of PCR primers using SYBR Green. Redesign probe or optimize probe binding step if primers are performing well
Probe exposed to light and been bleached	Ensure probe is stored at -20°C in the dark and returned to the freezer as soon as reaction setup.	
Non-specific amplification and / or primer-dimers	Annealing temperature too low	Increase annealing temperature in 2°C increments - use a thermal gradient if possible
	Poor primer design	Re-design primers using primer design software. It is good practice to try at least 2 primer pairs.
	RNA template contaminated with genomic DNA	Remove genomic DNA from RNA template with DNase I or use RT Enhancer with Verso kits. Design primers to span introns.
	RT reaction setup at room temperature	Setup the RT reactions on ice and transfer the RT reactions from ice to the reaction block, starting the RT protocol immediately
	Primers degraded	Check the integrity of the PCR primers by denaturing polyacrylamide gel electrophoresis

Fluorescence in the 'no template control' (NTC)	Reagents contaminated	Discard reagents and repeat assay with fresh reaction components
	Contamination occurred during reaction setup	Use barrier tips, screw-cap tubes and setup QPCR reaction in a DNA-free zone before adding the template in a separate location
Fluorescence in 'no RT control'	RNA template contaminated with genomic DNA	Remove genomic DNA from RNA template with DNase I or use RT Enhancer with Verso kits. Design primers to span introns.
Poor linearity of C_t values across dilution series (R value ≤ 0.998)	Too much nucleic acid in 'high copy number' assays	Use less than 500ng of template in each QPCR or QRT-PCR reaction
	Too little nucleic acid present in 'low copy number' assays	Increase the amount of template or increase PCR reaction efficiency by optimizing thermal protocol / re-designing primers
	Annealing temperature too low	Increase the annealing temperature in 2°C increments - use a thermal gradient if possible
	Reaction components not mixed thoroughly	Repeat assay ensuring all serial dilutions are vortexed for 15s and reaction components are mixed properly
	Poor template quality	Check the quality of the template preparation using spectrophotometry, microfluidics or PAGE
PCR efficiency is too high (>105%)	Primer-dimers bound to SYBR Green	Optimize thermal protocol ie. increase the annealing temperature in 2°C increments - use a thermal gradient if possible
	Serial dilutions not calculated properly	Repeat serial dilution using fresh sample material and calculate concentrations accurately
	Reaction not reproducible	Improve reproducibility by improving technique / optimizing thermal protocol / re-designing primers
PCR efficiency is too low (<90%)	Poor primer design	Re-design primers using primer design software. It is good practice to try at least 2 primer pairs.
	Annealing step too short	Increase annealing step in 3s increments up to 30s
	Annealing temperature too high	Decrease the annealing temperature in 2°C increments
	Extension time too short	Increase the extension time in 5s increments, up to 30s for amplicons of up to 500bp.
	Template contains inhibitors	Purify template or use different template extraction method and repeat the assay.
	Serial dilutions not calculated properly	Repeat serial dilution using fresh sample material and calculate concentrations accurately
	Amplicon too long	Amplicon should ideally be 100-150bp long and should not exceed 500bp
Fluorescent signal climbs and then falls sharply	Fluorescence increased so rapidly that baseline correction tilted curve forwards	Adjust baseline correction ie from cycles 3-15 to 3-10 or dilute template between 1:100 and 1:1000 and repeat
Amplification plot goes up, down and all around	Baseline has been set so when software applies data correction curves are distorted	Adjust baseline correction ie from cycles 3-15 to 3-10 or dilute template between 1:100 and 1:1000 and repeat
Amplification plot doesn't reach threshold	Baseline fluorescence is very high in 'high template' reactions	Manually adjust threshold so it crosses log-linear phase of each amplification plot or dilute template between 1:100 and 1:1000 and repeat
Amplification plot not exponential	Template contains inhibitors	Purify template or repeat assay using a 1:10 or a 1:100 dilution of template
Data plots very jagged	Data being collected at lowest detection limits of cycler	Smooth data by applying a moving average data correction algorithm or re-design assay