qPCR TROUBLESHOOTING GUIDE

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Problem	Possible Cause	Recommendation
No fluorescent signal trace and no product on agarose gel	Activation time too short	Ensure qPCR mix is activated for a minimum of 2min at 95°C before cycling.
	Error in protocol setup	Verify that correct reagent concentrations, volumes, dilutions and storage conditions have been used.
	Suboptimal primer/probe design	Use primer design software or validated primers/probes. Test assay on a control template.
	Incorrect concentration of primers/probe	Use primer concentrations between 200nM and 1µM. To optimize probe concentration, test the probe at several levels from 50 to 250 nM final concentrations in PCR with optimized levels of primers; generally, the probe should be at least 2-fold lower than the primer concentration.
	Template degraded	Re-isolate your template from the sample material or use freshly prepared template dilution. Verify the integrity of RNA using agarose gel electrophoresis.
	Primers/probe degraded	Use newly synthesized primers and/or probe
	Template contaminated with qPCR inhibitors	Perform a dilution series of the PCR template to determine whether the effect of the inhibitory agent can be reduced.
		Re-purify template and re-suspend it in DEPC-treated water.
	Template concentration too low	Increase concentration used - see protocol for ideal concentrations depending on type of template.
	Cycling conditions not optimal	Increase extension/annealing time, increase cycle number, reduce annealing temperature

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PCR product present on agarose gel but no fluorescent signal trace	Error in instrument setup	Check that the acquisition settings are correct during cycling
Non-specific amplification and/or primer-dimers	Inefficient reverse transcription	Extend reverse transcription time up to 30min and/or increase the temperature up to 55°C
	Suboptimal primer/probe design	Redesign primers and/or probe using appropriate software, or use validated primers/probes. Perform melt-curve analysis to check if primer-dimers are present / absent.
	Suboptimal primer/probe concentration	Use primer concentration between 100nM and 1µM. To optimize probe concentration, test the probe at several levels from 50 to 250 nM final concentrations in PCR with optimized levels of primers; generally, the probe should be at least 2-fold lower than the primer concentration.
	Annealing/extension temperature too low	Increase annealing/extension temperature up to 65°C or until primer-dimer/non-specific amplification products disappear.
	Suboptimal template concentration	Vary template concentration until non-specific products disappear
	Extension time too long	Reduce extension time to determine whether non-specific products are reduced.

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Late amplification trace	Inefficient reverse transcription	Extend reverse transcription time up to 30min and/or increase the temperature up to 55°C.
	Activation time too short	Ensure qPCR mix is activated for 2-3mins at 95°C before cycling, depending on type of template used - see protocol.
	Annealing temperature too high	Optimise annealing temperature in steps of 2°C using a thermal gradient.
	Extension time too short	Double extension time to determine whether the cycle threshold (CT) is affected.
	Template concentration too low	Increase concentration depending on template type - see protocol.
	Degraded or sheared nucleic acid template	Re-isolate your template from the sample material or use freshly prepared template dilution. Confirm the integrity of RNA using agarose gel electrophoresis.
		Do not store diluted template in water or at low concentrations.
	Suboptimal primer/probe design	Evaluate primer sequences for complementarity and secondary structure. Redesign primers/probe using appropriate software, or use validated primers.
	Suboptimal primer/probe concentration	Use primer concentration between 100nM and 1µM. To optimize probe concentration, test the probe at several levels from 50-250nM final concentrations in PCR with optimized levels of primers; generally, the probe should be at least 2-fold lower than the primer concentration.
	Assay Design - PCR target may be too long or may contain too much secondary structure leading to inefficient PCR	qPCR is most efficient when the PCR product is smaller in length. Select a target sequence with between 80 - 200bp for optimal results.
		Follow these guidelines to avoid secondary structure: Use an annealing temperature above the melting temperature (Tm) of any template secondary structure Avoid templates with long (>4) repeats of single bases Maintain a GC content of 50–60% Analyse secondary structure using the DNA mfold server created by Dr Michael Zuker (http://www.bioinfo.rpi.edu/applications/mfold) or an equivalent primer-design program

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High background signal or gradual increase in background fluorescence due to inefficient flurophore quenching	Partially degraded / unstable probe	Do not store diluted probes in water or at low concentrations.
		Do not store hybridisation probes at temperatures above –20°C (probes are prone to degradation at > -20°C, as bonds between the oligonucleotide probe and the conjugated fluorophore become labile).
		Check with the oligonucleotide manufacturer for the recommended dilution and storage conditions for the hybridisation probe.
		Aliquot probe stock into small volumes and thaw each aliquot only once (minimises multiple freeze-thaw cycles which degrade probes)
Signal in negative control	Poor laboratory technique	To minimise the possibility of contamination of PCR components by PCR product or other template, designate a work area exclusively for PCR assay setup.
		Use a solution of 10% bleach instead of ethanol to prepare the workstation area for PCR assay setup. Ethanol will only induce precipitation of DNA in your work area, while the 10% bleach solution will hydrolyse, as well as dissolve, any residual DNA.
		In general, follow these practices to minimize the risk of sample contamination: - Wear gloves - Use screwcap tubes - Use aerosol-resistant filter tips - Use calibrated pipets dedicated to PCR - Use PCR-grade water and use it only for PCR - Use a no-template control to verify absence of contamination - Prepare a master mix with sufficient volume to prepare all replicate samples

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Amplification of genomic DNA in no RT-control	Genomic DNA contamination of RNA used in RT-qPCR	Treat samples with purified RNase-free DNase before reverse transcription.
		It may also be helpful to design primers at splice junctions to avoid genomic DNA amplification.
Poor CT value reproducibility across replicate samples	Poor laboratory technique or imprecise pipetting	In general, follow these practices to improve replicate reproducibility: - Use aerosol-resistant filter tips - Use calibrated pipets dedicated to PCR - Use PCR-grade water and use it only for PCR - Prepare a master mix with sufficient volume to prepare all replicate samples - Add template to master mix before pipetting into reaction vessels - Avoid pipetting less than 5µl
	Inhibitors of PCR are carried over from sample preparation	Perform a dilution series of the PCR template to determine whether the effect of the inhibitory agent can be reduced.
		Take extra care with the nucleic acid extraction steps to minimize carryover of PCR inhibitors like phenol, detergents and proteases.
	Some primers are particularly sensitive to thermal cycling conditions, leading to poor reproducibility in amplification reactions.	Determine how your primers behave at different annealing temperatures by doing a gradient PCR.
	Air bubbles in reaction mix	Centrifuge reaction samples/plate prior to running on qPCR thermal cycler.