QPCR Optimization & Troubleshooting *Guide*

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Introduction

Whether you are beginning to develop a QPCR assay, have a QPCR assay you want to optimize, or are getting questionable results and don't know why, this guide is for you.

Simply bringing together all the necessary components for QPCR is often not enough to obtain accurate and consistent results. Following a protocol that instructs you how to combine your template with reagents before running them on your chosen instrument only covers the basics of real-time PCR. The science of real-time PCR comes from understanding how the nuances of this technique affect your results.

This quick reference guide is intended to educate you to gain a better understanding of these finer details, empowering you to optimize or troubleshoot your assay in order to achieve the best results possible on a consistent basis. It captures the knowledge that we have accumulated over 20 years manufacturing and optimizing PCR reagents and plastics for the life science industry.

Optimization *Guide*

Refer to this table before you begin your QPCR assay.

Use the information to ensure that you plan your protocol using best practice methodologies.

OPTIMIZATION PARAMETER	RECOMMENDATION
QPCR Plate	It is recommended that opaque white PCR plates are used for QPCR analysis. The white color virtually eliminates cross talk and improves the efficiency of fluorescent detection thereby increasing assay sensitivity and well-to-well consistency.
Template Quality	It is essential that the nucleic acid is sufficiently pure for QPCR analysis. Template contamination (ie. genomic DNA, protein, carbohydrates or organic solvents) can have a huge impact on assay reliability and reproducibility. Template quality should be determined by spectrophotometry (ie. Nanodrop), microfluidics or PAGE.
Amplicon Size	Ideally, the amplicon should always be between 100bp and 150bp to ensure the QPCR reaction efficiency is as close to 100% as possible. Good QPCR efficiency promotes assay reproducibility and sensitivity.
Primer Design	Given that PCR primers are a relatively cheap component of a QPCR assay, it is good practice to order and test at least 2 primer pairs for every new QPCR assay. This will maximize the chance of establishing a reliable, reproducible and sensitive assay.
Test Primers	Measure the reproducibility, specificity, sensitivity and dynamic range of your QPCR assay using SYBR Green chemistry across a template dilution series. Ideally, the efficiency of the QPCR reaction should be at least 90% and below 105%, while the assay reproducibility should be higher then r=0.998.
Efficient RT	Initially, the RT step should be performed as specified in the supplier protocol. However, the length and the temperature of the RT step can be optimized to increase the efficiency of the reverse transcriptase. The reverse transcriptase should be tested across a range of RNA concentrations to ensure assay linearity.
Hot-Start	Standard Thermo Scientific QPCR master mixes require a 15 minute heating step at 95°C to ensure the hot-start Taq is fully activated. This step cannot be shortened under any circumstances. A shorter heating step will impact assay reproducibility and sensitivity.
Thermal Protocol	Even if your assay has been optimized using an alternative supplier's mix, once the Thermo-Start mix is used it is advisible to start using the protocol recommended in the Thermo Scientific QPCR master mix. If assay optimization is required, the annealing temperature should be examined first.
Annealing Temperature	Test a range of annealing temperatures. Depending on the QPCR results, the annealing temperature should be increased or decreased in 2-3°C increments. This can be done in a single experiment using a thermal gradient. Alternatively, a range of annealing temperatures should be tested using multiple QPCR experiments.
Primer Concentration	Always start by using the primer concentration recommended in the master mix protocol. If optimization is required, try stepping the primer concentration up and down in 25mM increments. Optimizing primer concentration using a titration matrix can give improved results in rare circumstances but this is time-consuming.

Troubleshooting *Guide*

Refer to this table if you have performed a QPCR assay that resulted in sub-optimal results. Use this information to tweak the components, conditions, and reaction parameters in order to overcome erroneous results.

PROBLEM	POSSIBLE CAUSES	ACTIONS (Based on Thermo Scientific QPCR Master Mix Protocol)
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 (or 10 minutes in the Fast Protocol).
	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 the intercalating dye (eg. SYBR Green) or the dye conjugated to the fluorescent probe and that the ROX / fluorescein levels are correct.
	Error in setup	Check concentrations and storage conditions of the reaction components and then 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 in 25mM increments if necessary.
	Fluorescent data collected at wrong step	Ensure fluorescent data is collected at the appropriate step and in the correct channel.
	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	Once reaction set up is complete return your probe to the product packaging as soon as possible to mimimize exposure to light.

Troubleshooting *Guide*

PROBLEM	POSSIBLE CAUSES	ACTIONS (Based on Thermo Scientific QPCR Master Mix Protocol)
Non-specific amplification and / or primer-dimers	Annealing temperature too low	Increase annealing temperature in 2ºC increments - use a thermal gradient if possible.
	Poor primer design	Re-design primers using Primer3 or another QPCR primer design tool. 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 QRT-PCR kits. Design primers to span exon-exon boundaries.
	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 QRT-PCR kits. Design primers to span exon-exon boundaries.
Poor linearity of Ct 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 2ºC 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 2ºC 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.

Troubleshooting *Guide*

PROBLEM	POSSIBLE CAUSES	ACTIONS (Based on Thermo Scientific QPCR Master Mix Protocol)
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 are 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.

Innovative *QPCR Solutions*

Refer to our table of products for a start-to-finish solution of reagents, plates, and seals designed specifically to optimize QPCR results.

This system of products can dramatically improve the quality and reliability of your QPCR data.

	ABI						Bio-Rad				Strata-gene	Roche	Eppendorf
	7000/7300 7700/7900	7500	7900HT	7500 FAST	7900 FAST 7900HT FAST	StepOne StepOnePlus	iCycler MyiQ	iQ5	Opticon Opticon 2 Chromo4	Mini Opticon	Mx4000 Mx3000P Mx3005P	Lightcycler 480	Realplex
QPCR Plastics													
Thermo Scientific ABgene White QPCR Plates, 25 Plates (50 Plates with 384-well)													
96-Well	AB2400W	AB2400W	AB1900W	AB1900W	AB1900W	AB1900W	AB2400W	AB2400W	AB2800W	-	AB0900W	-	AB2800W
384-Well	-	-	AB1111W	-	-	-	-	-	-	-	-	-	-
Thermo Scientific EasyStrip Snap White QPCR Strip Tubes, 250 Strips with Attached QPCR Caps													
8-Strip	AB1502W	AB1502W	-	-	-	-	AB1502W	AB1502W	AB1502W	AB1502W	AB1502W	-	AB1502W
QPCR Reagents													
Thermo Scientific ABsolute Blue QPCR Master Mixes, 400 x 25uL (1 x 5mL)													
Probe	AB4139A	AB4319A	AB4139A	AB4319A	AB4319A	AB4319A	AB4137A	AB4137A	AB4137A	AB4137A	AB4137A	AB4137A	AB4137A
SYBR Green	AB4163A	AB4323A	AB4163A	AB4323A	AB4323A	AB4323A	AB4220A	AB4167A	AB4167A	AB4167A	AB4167A	AB4167A	AB4167A
Thermo Scientific ABsolute Blue Fast QPCR Master Mixes, 400 x 25uL (1 x 5mL)													
Probe	AB4328A	AB4330A	AB4328A	AB4330A	AB4328A	AB4330A	AB4326A	AB4326A	AB4326A	AB4326A	AB4326A	AB4326A	AB4326A
QRT-PCR Reagents													
Thermo Scientific Verso 1-Step QRT-PCR Mixes, 200 x 25uL (2 x 1.25mL)													
Probe	AB4101A	AB4102A	AB4101A	AB4102A	AB4102A	AB4102A	AB4100A	AB4100A	AB4100A	AB4100A	AB4100A	AB4100A	AB4100A
SYBR Green	AB4105A	AB4106A	AB4105A	AB4106A	AB4106A	AB4106A	AB4107A	AB4104A	AB4104A	AB4104A	AB4104A	AB4104A	AB4104A
Thermo Scientific Verso 2-Step QRT-PCR Kits, 200 x 25uL (2 x 1.25mL)													
Probe	AB4110A	AB4117A	AB4110A	AB4117A	AB4117A	AB4117A	AB4109A	AB4109A	AB4109A	AB4109A	AB4109A	AB4109A	AB4109A
SYBR Green	AB4113A	AB4116A	AB4113A	AB4116A	AB4116A	AB4116A	AB4114A	AB4112A	AB4112A	AB4112A	AB4112A	AB4112A	AB4112A
QPCR Plate Seal													
Thermo Scientific ABsolute QPCR Seal Ultra-Clear Adhesive Plate Seal, 50 Sheets													
AB-1170 (Compatible with all PCR plates) + Free Applicator AB-1391													

Larger pack sizes available. Please enquire.

Need help? Contact your local technical support team		
United States	(800) 235-9880, option #4	abgeneus.techservice@thermofisher.com
United Kingdom	01372 840 410	abgene.techsupport@thermofisher.com
France	01 60 92 48 68	abgene.techsupport@thermofisher.com
Germany	040 23 51 36 79	abgene.techsupport@thermofisher.com
Rest of the World	+44 1372 840 410	abgene.techsupport@thermofisher.com

How to order

United States

Direct: (PCR Reagents Only) Telephone (800) 874-3723 To fax an order (800) 842-5007 Order online: www.thermo.com/abgene	Fisher Scientific: (All Products) Telephone (800) 766-7000 To fax an order (800) 926-1166 Order online: www.fishersci.com

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