

Real-Time quantitative PCR Optimization Guide

INTRODUCTION

Real-Time quantitative PCR (qPCR) is a well-established method of detection and quantification of nucleic acids in biological samples. In conventional PCR, amplified DNA products are visualized via end-point analysis. In qPCR, a fluorescence detection method is employed such that the accumulation of the product can be measured in real-time as the reaction progresses, with quantification of the product after each PCR cycle. The applications of qPCR are far ranging, including gene expression analysis, strain typing, allelic discrimination, variant detection, analysis of genetically modified organisms and pathogen detection.

qPCR can also be coupled with reverse transcription (RT-qPCR) for the detection and quantification of RNA molecules. RT-qPCR can be a one-step (where reverse transcription and qPCR are carried out in a single step) or a two-step (where RNA is reverse transcribed to cDNA then this cDNA used as input to a separate qPCR reaction) process. Guidance on the design and optimization of RT-qPCR reactions can be found in the "RT-qPCR optimization guide".

qPCR analysis methods:

- Presence/absence qPCR analysis: Qualitative method to determine the presence or absence of a sequence. Applications include single nucleotide polymorphism (SNP) analysis and microbial detection.
- Relative quantification: Observed results are compared to controls used as reference material and can be expressed as higher or lower multiples of the control. This approach is extensively used for gene expression studies (in combination with reverse transcription).
- Standard curve analysis: Observed results are compared to a serially diluted standard curve of known concentrations to determine the absolute quantity of DNA in the sample. Standard curve analysis can be used to determine copy number variants for cancer phenotyping.

The following guide provides general advice for all three of the methods above. For more information on design, optimization and analysis of qPCR experiments, a number of published resources are available.¹⁻⁴

OPTIMIZATION

Detection method

Fluorescent PCR detection chemistries can largely be separated into two categories: intercalating dye-based and nucleic acid probe-based assays. Dye-based detection relies on DNA-binding dyes that fluoresce more strongly when bound to double-stranded DNA (dsDNA) than when unbound in solution such as SYBR® Green, EvaGreen® and SYTO™ dyes. Dye-based detection only requires the addition of primers to the PCR master mix and so can be cost effective and relatively simple to design. However, the intercalating dye will detect any dsDNA produced in the reaction, such as off-target and non-template amplification or primer-dimers, potentially resulting in inaccurate quantification. Denaturation (melt) analysis can be performed after the PCR to distinguish between on-target and non-specific products or for genotype analysis. Dye-based PCR cannot be multiplexed for quantitative detection because different amplicons cannot be distinguished during PCR cycling.

Probe-based assays use a sequence-specific oligonucleotide probe in addition to (or as part of) PCR primers such as TaqMan® probes, Molecular Beacons and Scorpions® probes. This improves assay specificity as only on-target amplified products are detected. Off-target amplification reactions are still possible and have the potential to lead to reduced fluorescent signal and loss in sensitivity. Furthermore, probes allow for multiplex assays where different amplicons can be targeted with probes designed with unique fluorophores and detected in qPCR equipment using separate filter channels. The requirement for these specific probes can increase costs of probe-based assays, but conversely, effective multiplexing can significantly increase throughput and reduce costs.

The most commonly used probes are hydrolysis probes (such as TaqMan probes) which rely on Förster Resonance Energy

Transfer (FRET) to prevent fluorescence from the 5'-dye moiety label via a 3'-quencher when the probe is intact. The probe is specific for sequences downstream of the qPCR primers and bind to ssDNA during the annealing step of qPCR cycling. During extension of the primer, the 5' to 3' nuclease activity of *Taq* DNA polymerase results in probe cleavage and spatial distancing of the dye and quencher, enabling the reporter (dye) molecule to emit fluorescent signal.

A passive reference dye can also be used in both dye-based and probe-based assays. ROX® (carboxyrhodamine) is an inert fluorescent dye that can be added to qPCR reactions. Unlike reporter dyes (e.g. SYBR Green), the ROX signal is not influenced

by the PCR reaction and thus is termed a 'passive reference dye'. ROX is a required additive for certain real-time PCR instruments for signal normalization to account for differences in the optical parameters at different plate well positions. Normalization to an internal reference dye can also normalize for differences in reaction volumes due to pipetting errors or evaporation and reduce the variability between technical replicates. Different types of real-time PCR systems that require a passive reference standard have different optimal concentrations of ROX, mainly due to the different optical configurations of each system, particularly the type of excitation source and optics used.

Method	Advantages	Disadvantages
Dye-based detection	<ul style="list-style-type: none"> ■ Economical method for detection and quantification of PCR products ■ Ideal for optimization of qPCR reactions ■ Use conventional PCR primers ■ Useful for generating melt curves for genotype analysis 	<ul style="list-style-type: none"> ■ Bind non-specifically to dsDNA ■ Cannot be used for multiplexing
Probe based	<ul style="list-style-type: none"> ■ Greater target specificity than dye-based ■ Greater specificity for mis-matches ■ Ability to multiplex reactions 	<ul style="list-style-type: none"> ■ Probes can be expensive ■ Complicated assay design ■ May require specific primers (e.g. Scorpion probes) ■ Probes unavailable for melt curve analysis (hydrolysis probes e.g. TaqMan®)

Table 1 Overview of detection methods for qPCR.

DNA polymerase

The choice of a thermostable DNA polymerase can determine amplification efficiency, specificity and sensitivity. *Taq* DNA polymerase is most commonly utilized for qPCR nucleic acid amplification, but like all polymerases it has residual activity at low temperatures. During reaction set-up, primers can anneal to the template non-specifically, allowing synthesis of non-specific products that can lead to unwanted amplification during PCR cycling. This can be avoided with the use of a 'hot-start' enzyme. Hot-start enzymes employ a mechanism of enzyme inactivation during reaction set up. This can be a monoclonal-antibody or chemical moiety which undergoes a change in structure upon heating. PCR protocols with a hot-start enzyme typically are activated during an initial denaturation step at $\geq 95^{\circ}\text{C}$ before thermocycling. All Quantabio PCR and qPCR enzymes use antibody hot-start technology where multiple monoclonal antibodies inactivate the *Taq* polymerase at room temperature. The antibody hot-start mechanism can be advantageous compared to chemical hot-start technology. Antibodies ensure complete enzyme inactivation below 55°C ,

then upon heating, they are irreversibly denatured and do not inhibit PCR.

Although *Taq* polymerase is widely used in molecular biology, there are a vast array of alternative enzymes or mutants available. Some thermostable polymerases include proof-reading activity to correct synthesized DNA. As amplicons for qPCR are typically short, a high-fidelity enzyme is not usually necessary, unless downstream sequencing of PCR products is intended. When amplification detection is carried out using hydrolysis probes, the DNA polymerase must have the 5' to 3' nuclease activity requisite for probe cleavage.

Primer and amplicon design

The design of highly specific primers and probes is a critical parameter for successful real-time PCR. The use of computer aided primer design programs is encouraged in order to minimize the potential for internal secondary structure and complementation at 3'-ends within each primer, the primer pair, and primer/probe combinations.

General parameters for primer design are listed below. More details are given in our PCR Primer Design Guide (https://www.quantabio.com/media/contenttype/MK-TN-0002_REV01_PCR_Primer_Design_0918.pdf).

- Primers should be between 18-24 bases in length
- Maintain GC-content of 50 – 60%, avoiding repeats of more than three G or C bases
- Place Gs or Cs at 3' ends of primers
- Melting temperature (T_m) between 57-61°C, with <5°C difference between primers
- Avoid regions of strong secondary structure
- Avoid self-complementary primers

When using probe-based detection, general parameters for probe design are given below. Pre-designed probes are available for many gene targets.

- <30 bases in length
- T_m 5 – 10°C higher than that of primers
- GC-content 30 – 80%
- Probes can be designed to bind to either strand and should be positioned close to one of the primers without overlapping the primer binding site
- Avoid a G at the 5' end to prevent quenching of the 5' fluorophore
- Reporter and quencher combination to suit the capabilities of the qPCR instrument

General parameters for amplicon selection:

- 65 – 200 bp in length. Some PCR master mixes such as PerfeCta® SYBR Green SuperMix can readily amplify amplicons up to 500 bp but amplification of smaller targets will be more efficient
- Avoid secondary structure
- Avoid templates with long (>4) repeats of single bases
- Maintain GC-content of 50 – 60%

Primer/probe concentration

Optimal PCR amplification results may require titration of primer and probe concentration. A final concentration of 300 – 400 nM for each primer and 250 nM probe is effective for most reactions, particularly if the target is abundant and maximum sensitivity is not required. Primer concentration can be optimized between 100 and 900 nM to improve reaction efficiency and specificity. Increasing the concentration of the primer that initiates synthesis of the target strand complementary to the probe can improve fluorescent signal for some primer/probe systems. When using dye-based detection, lower primer concentrations can minimize non-specific amplification. If maximum sensitivity is not required, probe concentration can be varied between 50 – 250 nM to reduce assay cost.

Component	Recommended concentration	Optimization range
Primers	300 – 400 nM	100 – 900 nM
Probe	250 nM	50 – 250 nM
Mg ²⁺	3 mM	3 – 6 mM
Template input	10 – 1000 copies	1 pg – 100 ng cDNA 100 pg – 100 ng gDNA

Table 2 General guidelines for concentrations of qPCR reaction components.

Template input

Quantitative PCR can only be used reliably when the template region of interest can be amplified completely without blocks, nicks or breaks. Therefore it is critical that input DNA or cDNA is of suitable quality for amplification, without lesions or abasic sites. In addition, some additives or components from DNA extraction or carryover inhibitors from the sample source can inhibit the DNA polymerase.

qPCR allows detection of input material over a wide dynamic range covering 6 to 8 orders of magnitude. The higher the complexity of the input DNA, the fewer the copies of the target that will be present in a given DNA quantity. In general, recommended inputs are approximately 100 pg – 100 ng gDNA or cDNA corresponding to 1 pg – 100 ng total RNA. For low complexity templates such as plasmid DNA, viral (e.g. lambda

phage) DNA or bacterial artificial chromosomes, a lower input range of 1 pg – 10 ng per reaction is recommended. Increased input can improve sensitivity but also increases the concentration of potential PCR inhibitors in the reaction. The use of an inhibitor-resistant master mix such as Quantabio's ToughMixes can avoid PCR inhibition.

Generally, two-step RT-qPCR protocols benefit from RNase H+ activity when long cDNA strands are synthesized. In one-step RT-qPCR reactions, where short cDNA strands are synthesized more often, template suppression is unusual. Quantabio offers both one and two-step RT-(qPCR) options with RNase H+ activity (e.g. qScript® cDNA SuperMix) or reduced RNase activity (e.g. qScript XLT cDNA SuperMix and qScript XLT 1-Step RT-qPCR ToughMix®). In addition, Quantabio one-step RT-qPCR master mixes benefit from accessory proteins which further prevent any suppression of cDNA synthesis by the RNA template.

Cycling conditions

The recommended PCR cycling conditions for the selected PCR master mix used should be followed. Hot-start enzymes are recommended for qPCR to prevent non-specific amplification during reaction set up and so an initial denaturation step is usually required. For antibody mediated hot-start enzymes, a 30 s initial denaturation step at 95°C is typically sufficient for full enzyme activation. This hot start step can also be beneficial to fully denature long templates or templates with significant secondary structure. Initial denaturation times greater than 3 min are usually not required when amplifying cDNA templates. Amplification of genomic DNA or supercoiled plasmid DNA targets may benefit from a prolonged initial denaturation step (5-10 min) to fully denature and fragment the template. This minimizes the potential for renaturation of long fragments and/or repetitive sequence regions that can impair the amplification of the target sequence.

Following initial denaturation, PCR proceeds during cycles of denaturation, annealing and extension. During denaturation, high temperature incubation is used to melt dsDNA into single strands and to relax secondary structure. High GC-content DNA or DNA with significant secondary structure may require a longer denaturation time. The annealing step, where complementary sequences hybridize, is a key point of assay optimization as the annealing temperature can determine the specificity of primer binding. The annealing temperature can be calculated based on the melting temperature of primers (typically 5°C below

the T_m of the primers) and optimized empirically for a given primer set with a gradient PCR. Synthesis of the complementary DNA strand takes place during the extension step, typically at around 70 - 72°C for optimal activity of the DNA polymerase.

Extension time is dependent upon amplicon length and the minimal data collection time requirement for the qPCR instrument. A 68 to 72°C extension step of 30 s is suitable for most applications. However, amplicons greater than 200 bp may require longer extension times. For small amplicons, annealing and extension steps can be combined if the melting temperature of primers is suitable although some primer sets may require a 3-step cycling protocol for optimal performance.

As the change in fluorescence will vary across the cycle with different detection technologies it is also important to detect fluorescence at the correct part of the PCR cycle for the technology used: at the end of the extension step for dye-based assays and hydrolysis probes, and during the annealing step for hybridization probes. The use of an elevated temperature (80°C) for data collection is not recommended. While this technique can be used to mask the detection of primer-dimer and/or other non-specific products, it does little to improve assay specificity or sensitivity and is not a substitute for effective primer design.

Uracil N-glycosylase (UNG)

For laboratories that routinely carry out qPCR, carryover PCR product presents a contamination risk. If qPCR reactions are carried out with dUTP in place of dTTP in the dNTP mix, all PCR products will contain uracil instead of thymine bases. Therefore, input DNA can be treated with uracil N-glycosylase to specifically digest uracil containing DNA (carryover PCR product). The template DNA contains thymine, not uracil, and so will not be degraded. Some PCR master mixes (e.g. PerfeCтa qPCR FastMix, UNG and PerfeCтa qPCR ToughMix, UNG) contain a heat-labile UNG enzyme to streamline this process into the qPCR workflow. The qPCR reaction is set up as normal, using the master mix containing UNG and dUTP. An incubation step at 42°C is added at the start of the protocol where UNG will digest any contaminating uracil-containing DNA from previous qPCR reactions carried out with dUTP. The UNG is then deactivated during the high temperature initial denaturation step of the qPCR reaction preventing any degradation of the current qPCR product, ensuring only signal from the intended template is detected.

Multiplexing

A key advantage of probe-based qPCR assays is the ability to multiplex to interrogate multiple targets in a single reaction, increasing throughput and reducing sample and reagent usage. To be successful, PCR multiplexing must be capable of producing sufficient amplified product for the detection of all of the target sequences. Results obtained from multiplex reactions should be verified to confirm that the same results would be obtained if the reactions were performed individually.

When the abundance of targets varies 'multiplex PCR saturation' can occur, where the amplification of a more abundant gene saturates the DNA polymerase, suppressing the amplification of less abundant genes. This can be avoided by reducing the primer concentration for the abundant target ('primer limitation'). Specialized multiplex master mixes are available to deliver dynamic range and sensitivity to multiplexed qPCR that is comparable to that for singleplex qPCR probe assays without the need for limiting or varying primer concentrations, such as PerfeCta Multiplex qPCR ToughMix.

Additionally in multiplex PCR, there is a risk of unexpected interaction between primers from different assays. The design of highly specific primers and probes is a critical and challenging aspect of successful multiplex qPCR. Each primer and probe should have similar thermodynamic properties to support efficient PCR amplification using a common temperature cycling program for all amplicons. The use of computer-aided primer design programs is encouraged in order to minimize the potential for internal secondary structure and complementation at 3'-ends within each primer, primer pair, and primer/probe combinations. Amplicon size should be consistent for each target sequence and limited to approximately 65 – 100 bp.

For multiplex PCR, there are also further considerations for probe dye (fluorophore) selection. The qPCR instrument used

must be capable of detecting the emission spectrum of each dye selected. The settings for excitation and emission filters for real-time detection systems also vary between manufacturers, so the instrument may need to be calibrated for the dyes chosen. The robust fixed optics in the Q qPCR instrument require no calibration for detection of various dyes, removing this step from optimization of multiplex PCR. A list of dyes compatible with the Q qPCR instrument is available (https://www.quantabio.com/wp-content/uploads/2021/12/MK-BL-0005_REV_01_Q_qPCR_Cycler_Dye_Detection_1018.pdf).

Each probe used to detect individual targets should be labeled using unique dyes with appropriate excitation wavelengths and little to no overlap in their emission spectra. Use of dyes with discrete fluorescence excitation and emission optima improves the accuracy of the multi-componenting or dye deconvolution algorithms employed by the real-time PCR analysis software. Dyes that have an overlapping spectra with ROX (e.g. JUN or ABY) should be avoided if using an instrument that uses ROX as a passive reference dye (or a ROX containing mastermix). Probes should also be designed with minimal background fluorescence, by using appropriate, non-fluorescent quencher compounds. Online tools are available to optimize fluorophore/quencher and dye combinations such as IDT PrimeTime™ Dye Selection Tool.⁵

The total fluorescence intensity should also be considered. For example, a fluorophore with high fluorescent signal intensity (such as FAM) is a good dye choice to detect low-copy transcripts. Whereas a fluorophore with lower signal intensity should be considered for high-copy transcripts such as house-keeping genes.





Dye	Excitation	Emission	Channel
FAM	494	515	
CAL Flour Orange 560	540	561	
CAL Flour Red 610	590	610	
Quasar 670	647	667	

Table 3 Example dye combinations for a 4-plex qPCR reaction on the 4 channel Q qPCR instrument. Many more dye combinations are available.

PCR inhibitors

Enzyme inhibitors can be present in gDNA or cDNA samples. The use of organic solvents (e.g. ethanol) and chaotropic salts (e.g. guanidine) are necessary for DNA and RNA extraction and purification, but these can inhibit enzymatic reaction either by direct interaction with the enzymes or with reaction co-factors. In addition, where samples contain different levels of inhibitors, sample comparison may not be accurate. The use of a DNA/RNA isolation method that minimizes inhibitor carryover can alleviate these issues, but this is not always possible. Moreover, in clinical settings there is a drive towards using unprocessed patient samples for reaction input. In such cases, choosing an inhibitor-resistant enzyme formulation is vital.

Quantabio's engineered application-specific enzymes combined with optimized ToughMix buffer formulations allow superior performance in the presence of inhibitors in crude samples extracted from environmental specimens, plant tissues or animal tissues. In some cases, time consuming and expensive sample purification can be bypassed enabling accurate and reliable qPCR directly from crude lysates. ToughMix reagents are available for probe-based detection only. The ToughMix buffer is not compatible with SYBR Green dye, however AccuStart II PCR ToughMix has been successfully used in combination with EvaGreen (Biotium) for intercalating dye-based qPCR detection with difficult samples.⁶ In addition, AccuStart II PCR ToughMix has been demonstrated as an effective reagent for high-resolution melt analysis in combination with LCGreen® Plus (BioFire Defence).⁷

Reaction efficiency

Reaction efficiency is best assessed through the generation of a standard curve. A standard curve is generated by creating a dilution series of sample nucleic acid and performing real-time PCR. Samples used to generate the standard curve should match (as closely as possible) those that will be used for the experiment and the dilution range, or dynamic range analyzed for the standard curve, should span the concentration range expected for the experimental samples.

The slope of the log-linear phase of the amplification reaction is a measure of reaction efficiency. On a plot of Log (input quantity) versus the threshold cycle (C_T) value for each dilution of the standard curve, the efficiency of the PCR reaction can be calculated according to the following equation:

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1$$

A PCR efficiency of 100% corresponds to a slope of -3.32 and reactions should have an efficiency as close to this as possible to ensure accurate and reproducible results. PCR efficiency can be influenced by experimental factors such as the length, secondary structure and GC-content of the amplicon, the use of non-optimal reagent concentrations or the presence of PCR inhibitors. Typically, a reaction efficiency between 90 and 110% (corresponding to a slope between -3.58 and -3.10) is acceptable.

Controls

For accurate qPCR data analysis, each experiment needs to be set up with multiple replicates and controls.

- Replicates: For each experimental and control sample to be compared, at least three technical replicates are recommended to minimize errors from pipetting and reaction setup.
- No RT control: When using cDNA input (i.e. for two-step RT-qPCR), a 'no reverse transcriptase' control should be carried out for each reverse transcription reaction to identify erroneous signal from contaminating gDNA.
- No template control: During qPCR, a 'no template control' should be set up for each assay carried out to identify possible cross-contamination during sample preparation.
- Reference genes: For gene expression assays, multiple reference genes should be analyzed for stable gene expression. It is important to use a reference gene with an expression pattern that is unaltered by the test condition.

TROUBLESHOOTING

Issue	Causes	Troubleshooting
Primer-dimer formation	Primers with high complementarity or long primers can preferentially bind to each other rather than the template.	<ul style="list-style-type: none"> Optimize annealing temperature (typically increase) Decrease primer concentration Redesign primers with lower T_m Redesign primers with lower complementarity or shorter primers Decrease Mg^{2+} concentration
Signal in no-RT control	Non-specific amplification of contaminating DNA	<ul style="list-style-type: none"> Use good laboratory practice to avoid contamination during reaction set up Use master mix with dUTP/UNG to prevent carry over contamination from previous PCR Swap out reagents for a fresh aliquot where possible Use a hot-start enzyme
	Primer dimerization	<ul style="list-style-type: none"> See above "Primer dimer formation"
Poor amplification efficiency or no amplification	Not enough input of target	<ul style="list-style-type: none"> Optimize reverse transcription reaction for cDNA input Increase input DNA concentration Improve extraction method
	Inhibition of reporter dye	<ul style="list-style-type: none"> Improve extraction method Use an alternative dye
	DNase I carryover from treatment of RNA sample can degrade cDNA or amplified DNA during PCR step	<ul style="list-style-type: none"> If treating RNA sample with DNase, ensure full deactivation of DNase or sufficient washing with column based methods before reverse transcription
	Inhibition of qPCR	<ul style="list-style-type: none"> Use enzyme engineered to overcome inhibitors Use master mix with inhibitor resistant formulation Dilute input DNA
	Insufficient primer concentration	<ul style="list-style-type: none"> Increase primer concentration Multiplex PCR may require different primer concentrations for different targets
	Cycling conditions	<ul style="list-style-type: none"> Optimize denaturation time and temperature to fully denature high GC-content DNA Optimize annealing temperature with gradient PCR Optimize extension time for length of target
	Poor detection of fluorescent signal	<ul style="list-style-type: none"> Ensure qPCR instrument is set-up correctly for signal collection
	Poor signal-to noise ratio	<ul style="list-style-type: none"> Ensure probes are stored correctly to prevent degradation of probe and release of free dye (high background signal) or photobleaching
	Poor assay design	<ul style="list-style-type: none"> Check sequence databases for variants of gene of interest Check position of primers on target gene (e.g. intron or exon) Check primer design for secondary structure or complementarity
Multiple peaks in melt curve	Non-specific amplification of contaminating DNA or non-specific primer binding	<ul style="list-style-type: none"> Use a hot-start enzyme Change primer design to prevent off-target priming Reduce extension time Optimize annealing temperature with gradient PCR Use good laboratory practice to avoid contamination during reaction set up Use mastermix with dUTP/UNG to prevent carry over contamination from previous PCR Primers can degrade and lose specificity if stored incorrectly Check for DNA contamination in RNA sample Increase temperature of reverse transcription reaction Use gene specific priming for reverse transcription

Issue	Causes	Troubleshooting
qPCR Reaction efficiency >110%	Inhibition of reaction	<ul style="list-style-type: none"> Use enzyme engineered to overcome inhibitors Use master mix with inhibitor resistant formulation Dilute input RNA (one-step RT-qPCR) or cDNA (two-step RT-qPCR)
qPCR Reaction efficiency <90%	Suboptimal reagent concentrations Suboptimal PCR cycling	<ul style="list-style-type: none"> Vary concentration of primers, Mg²⁺ or polymerase Check primer design, T_m of primers should be within 5°C of each other Optimize annealing temperature with gradient PCR Optimize extension time according to polymerase processivity rate Increase initial denaturation and final extension times for PCR

SUMMARY OF OPTIMIZATION PROCESS



PRODUCT SELECTION

This table provides broad guidance on Quantabio reagent selection for qPCR experiments. It is not exhaustive so further products and guidance can be found at www.quantabio.com.

Application	Recommended product
Dye-based detection	
Dye-based qPCR	PerfeCtra SYBR Green SuperMix
Probe-based detection	
Genotyping	AccuStart Genotyping ToughMix
General qPCR	PerfeCtra FastMix II
qPCR from crude/difficult input samples	PerfeCtra qPCR ToughMix
Multiplex qPCR, even from crude/difficult input samples	PerfeCtra Multiplex qPCR ToughMix
Mixes containing dUTP/UNG	PerfeCtra qPCR FastMix UNG PerfeCtra qPCR ToughMix, UNG

Table 4 General guidelines for Quantabio reagent selection.

References

1. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical chemistry*. 2009;55(4):611-622.
2. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research*. 2001;29(9):e45.
3. T Nolan, J Huggett, E Sanchez. Good practice guide for the application of quantitative PCR (qPCR). LGC. 2013. Available at: <https://www.gene-quantification.de/national-measurement-system-qpcr-guide.pdf>. Accessed 07/20/2020.
4. Taylor SC, Nadeau K, Abbasi M, Lachance C, Nguyen M, Fenrich J. The Ultimate qPCR Experiment: Producing Publication Quality, Reproducible Data the First Time. *Trends in biotechnology*. 2019;37(7):761-774.
5. IDT. IDT PrimeTime Dye Selection Tool. Available at: <https://eu.idtdna.com/site/order/qpcr/primetimeprobes/multiplex>. Accessed 07-26-2022.
6. Fornasaro S, Esposito A, Florian F, et al. Spectroscopic investigation of faeces with surface-enhanced Raman scattering: a case study with coeliac patients on gluten-free diet. *Analytical and bioanalytical chemistry*. 2022;414(11):3517-3527.
7. Oh Y, Barbey CR, Chandra S, et al. Genomic Characterization of the Fruity Aroma Gene, FaFAD1, Reveals a Gene Dosage Effect on γ -Decalactone Production in Strawberry (*Fragaria x ananassa*). *Frontiers in plant science*. 2021;12:639345.

Trademarks: PerfeCtra®, qScript® and ToughMix® are registered trademarks of Quantabio, LLC. SYBR®, SYBR® Green, SYTO™, and ROX® are registered trademarks of Thermo Fisher Scientific. EvaGreen® is a trademark of Biotium, Inc. TaqMan® is a registered trademark of Roche Molecular Systems, Inc. Scorpion® is a registered trademark of Qiagen, Inc. PrimeTime™ is a trademark of Integrated DNA Technologies, Inc. LCGreen® is a trademark of BioFire Diagnostics, Inc.

Quantabio products are intended for molecular biology applications. The products are not intended for the diagnosis, prevention or treatment of a disease.

MK-AN-0014 REV 01 qPCR Optimization Guide 1022