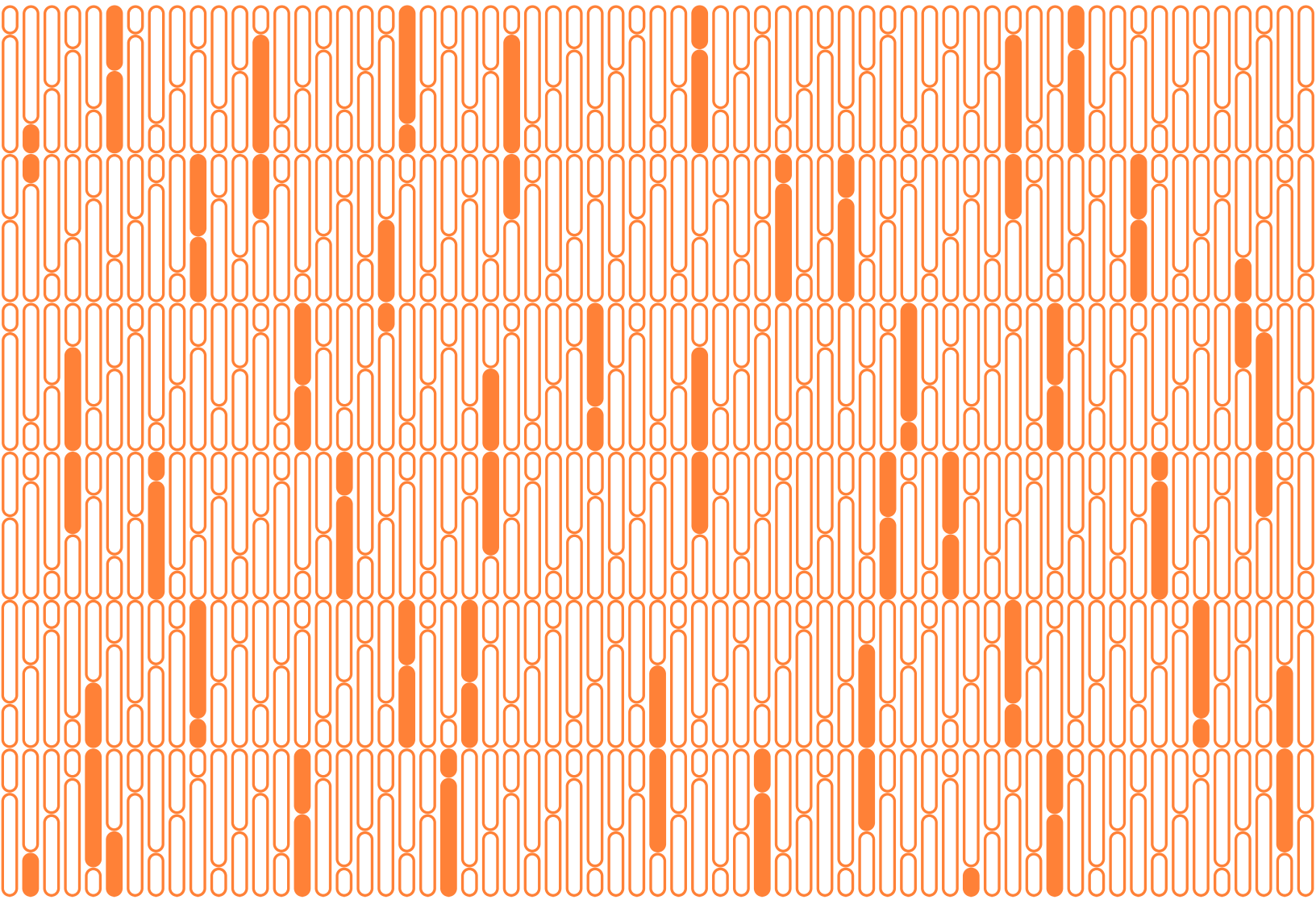
Real-time qPCR assay design guide

Guidance for gene expression experiments

First edition



IDT® INTEGRATED DNA TECHNOLOGIES

YOUR ADVOCATE FOR THE GENOMICS AGE

www.idtdna.com

Real-time qPCR assay design guide

Guidance for gene expression experiments

First edition

Managing editors and contributors

Nicola Brookman-Amissah, PhD[1]; Hans Packer, PhD[1]; Ellen Prediger, PhD[1]; Jaime Sabel[1]

Contributors

Stephen Gunstream[1]; Jan Hellemans, PhD[2]; Aurita Menezes, PhD[1]; Sharon Rouw[1], Brendan Owens[1]; Scott Rose[1], PhD[1]; Rick Sander[1]; Jo Vandesompele, PhD[2]

1 Integrated DNA Technologies; 2 Biogazelle NV, Belgium; Ghent University, Belgium

Table of contents

1. Introduction

This guide will advise you on designing effective quantitative PCR (qPCR) assays for successful gene expression studies. It includes expertise from our scientists with recommendations and tips for assay design, as well as lists of useful reagents and free online software programs that can be used to aid primer and assay design.

Unlike endpoint, reverse transcription–PCR (RT-PCR), used for determining the presence or absence of a particular gene product, real-time PCR, qPCR, or RT-PCR (referred to as qPCR throughout this guide) can provide a measure of the starting copy number and detect small differences in expression levels between samples. qPCR involves fluorescent reagents, which allow investigators to observe PCR product accumulation over the entire amplification and eliminate the need to run a gel, reducing the duration of the process and the chance of contamination. Amplification and quantification occur simultaneously using specialized thermal cyclers equipped with fluorescence detectors.

While endpoint PCR is robust and almost always produces "a result", ensuring that qPCR is quantitative, accurate, and reproducible requires thoughtful assay design, setup, and optimization. Poor qPCR assay design—imperfect reaction conditions (e.g., choice of master mix, primer or probe T_m), presence of inhibitors or underlying single-nucleotide polymorphisms (SNPs)—can lead to suboptimal amplification and extensive troubleshooting.

Even use of previously identified assays (e.g., from the literature) that were not validated under your specific reaction conditions (e.g., RNA extraction method, assay reagents, thermal cycler), can require repeating experiments, wasting valuable time and resources, including precious samples [1].

To achieve reliable, interpretable results from qPCR, the following important factors must be considered:

- Primer and probe design are crucial to the success of the experiment.
- The real-time PCR instrument will dictate certain parameters of the experiment; importantly, some instruments are not compatible with some fluorescent dyes.
- If you are running a multiplex experiment, additional considerations will need to be incorporated—particularly in the assay design and choice of dyes.
- As a final step before the reaction is set up, determine the controls that you will run. Include both positive and negative controls, and be sure to calculate those extra reactions into your total number of reactions.
- Always use RNase- and DNase-free reagents, check their expiration dates, and verify their concentrations.

Let's get started.

2. 5′ Nuclease assay selection and design

a. General design considerations

Know your gene: A well-designed assay begins with an understanding of the gene of interest, including knowledge of the transcript variants and their exon organization. Use databases such as Ensembl (European Molecular Biology Laboratory's European Bioinformatics Institute) or GenBank® (US National Center for Biotechnology Information, NCBI) databases to identify exon junctions, splice variants, and locations of SNPs. For genes that have multiple transcript variants, align related transcripts to understand exon overlap using a program, such as Clustal (Science Foundation Ireland and University College Dublin), or online tools, such as the Genome Data Viewer (NCBI). For transcript-specific designs, target primers and probes within exons unique to the transcripts of interest, and use BLAST® tool (Basic Local Alignment Search Tool; NCBI) to ensure primer and probe sequences do not occur in related transcripts or cross-react with other genes within the species (see *Ensure specificity* section below). For splice-common designs, target primers and probes within exons found across all transcript variants.

Manage SNP positioning: With the increased focus on high-throughput sequencing, the number of identified SNPs in the human genome is rapidly increasing. In the human genome, SNPs are present in at least 1% of the population and occur on average once every few hundred bases [2]. Given the high frequency of SNP occurrence, it is unrealistic to try to avoid SNPs altogether when designing PCR/qPCR assays. However, performing PCR using primers and probe sequences that overlie SNP sites can dramatically impact a reaction or can have little to no impact at all. Specifically, the position of SNPs underlying a primer or probe can influence primer and probe T_m, efficiency of polymerase extension, and even target specificity. To obtain the most accurate data, it therefore becomes important to know how your assay designs overlie SNPs and manage this positioning (see the IDT DECODED newsletter article, *Considering SNPs when designing PCR and qPCR assays*).

Ensure specificity: Ensure that both the primers and the probe are specific to the target and not complementary to other sequences. Use BLAST to analyze the sequences to ensure their specificity. The BLAST tool finds regions of local similarity between sequences. Enter an accession number or the sequence in FASTA format and compare it to the genome of a specific species or to all BLAST databases. BLAST allows searches against nucleotide or protein databases and provides the statistical significance of the matches (for more information, see the IDT DECODED newsletter article, *Tips for using BLAST to locate PCR primers*; also see Section 6d).

IDT predesigned qPCR assays

PrimeTime Predesigned qPCR Assays are generated by a design algorithm that accounts for these general design parameters. The primers and probe sequences have been analyzed by the BLAST tool and assessed for underlying SNPs. See Section 5a for more information.

Working with limited samples or low abundance targets: For two-step RT-qPCR protocols, the input amount of cDNA used for qPCR can be regulated to increase the amount of target available for detection. This is useful when working with low abundance targets when sample is not limited.

When sample is limited, preamplification of RNA (by linear, isothermal amplification) or first-strand cDNA, before qPCR, can increase the amount of detectable target for low abundance transcripts from minute amounts of sample. This extra step is often added when performing single-cell analysis or working with clinical samples, fine needle biopsies, laser captured microdissection samples, or FACS-generated cells.

MIQE publications

The information in this guide is designed to follow the standards outlined in *The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments* [3]. The purpose of the MIQE guidelines outlined in this publication is to provide a minimum set of requirements for conducting qPCR experiments that enable other researchers to replicate findings. We at IDT recommend that investigators using RT-qPCR review those guidelines together with this guide, to aid assay performance, reproducibility, and publication of experimental data.

Two updated publications, *MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments* [4] and *Primer sequence disclosure: a clarification of the MIQE guidelines* [5], provide additional information on sample handing, experimental conditions, normalization to reference genes, and data analysis.

b. Primers and probes

It is important to give careful consideration to the locations and characteristics of primers, probes, and amplicons before starting any real-time PCR experiment. Particularly crucial for primers and probes is ensuring you have an appropriate melting temperature (T_m), which determines the conditions under which the primers and probe will bind to their target sequence. Oligo length and GC content will both affect T_m. Our recommendations for primers, probe, and amplicon specifications are provided in the following text and summarized in Table 1. In addition, we provide a suite of free, online tools (SciTools programs) that aid in primer and probe selection and design. See Section 6 for more information.

	Primers		Probe		Amplicon	
	Range	Ideal	Range	Ideal	Range	Ideal
Length	18–30 nt	22 nt	20–28 nt*	24 nt	70–150 bp	100 bp
Melting temperature	60–64°C	62°C	66–70°C	68°C	NA	NA
GC content	35–65%	50%	35–65%	50%	NA	NA

* For probes that do not contain minor groove binder (MGB) T_m-enhanced properties

Table 1. Recommended specifications for primers, probes, and amplicons. These specifications are based on a final reaction composition of 50 mM KCl, 3 mM $MgCl_2$, and 0.8 mM dNTPs.

i. Primers

T_m: Typically, an annealing temperature (T_a) of 60°C is used during PCR. The optimal melting temperature (T_m) of the primers is between 60 and 64°C, with an ideal temperature of 62°C, which is based on the average conditions and factors associated with the PCR. The melting temperature of the 2 primers should not differ by more than 4°C for both primers to simultaneously bind their targets and efficiently amplify the product.

Length: Aim for primer lengths of 18–30 bases with a balance among the melting temperature, purity, specificity, and secondary structure considerations.

GC content: Ensure that the primers are specific to the target and that they do not contain regions of 4 or more consecutive Gs [6]. The GC content should be within the range of 35–65%, with an ideal content of 50%, which allows complexity while still maintaining a unique sequence. Avoid sequences that may create secondary structures, self-dimers, and heterodimers; use programs such as the IDT OligoAnalyzer Tool (Section 6e) to find potential sites that are likely to form these structures.

ii. Probes

T_m: The melting temperature of the probe should be 6–8°C higher than the primers and should fall within the range of 66–70°C for a standard two-step protocol. If the melting temperature is too low, the probe will not bind to the target. In this case, the primers may amplify a product, but as the probe is not bound to a target, it will not be proportionally degraded and, thus, will be unable to provide the fluorescence that is necessary to detect the product.

Length: The length of a single-quenched probe should be 20–30 bases to achieve an ideal T_m without increasing the distance between the dye and quencher, such that the quencher will no longer absorb the fluorescence of the dye (i.e., probes with a single terminal quencher that are longer than 30 bases may perform poorly due to the distance between the quencher and dye). Double-quenched probes, such as those that include the IDT ZEN or TAO molecules as a secondary, internal quencher, allow longer probes to be used, while providing strong quenching and increased signal. See Section 5c for more information on double-quenched probes.

GC content: Aim for a GC content of 35–65%, and avoid a G at the 5′ end to prevent quenching of the 5′ fluorophore. As with the primers, avoid sequences that may create secondary structures or dimers.

Location: Ideally, the probe should be in close proximity to the forward or reverse primer, but not overlap, although this is not absolutely necessary. Probes can be designed to bind to either strand of the target.

iii. Amplicons

Length: Design amplicons of 70–150 bp, which will allow the primers and probe to compete for hybridization and provide a sequence that is long enough for all components to bind. This length is most easily amplified using standard cycling conditions. Longer amplicons of up to 500 bases can be generated, but cycling conditions will need to be altered to account for the increased extension time. Amplicons or assay designs should span an exon–exon junction to reduce the possibility of genomic contamination.

T_m: Calculate all melting temperatures under real-time PCR conditions. Standard parameters for qPCR are 50 mM K^+, 3 mM Mg^{2+}, and 0.8 mM dNTPs; however, they can vary widely from this, particularly with respect to Mg^{2+} concentration. See the next section for more information on calculating melting temperature.

iv. Melting temperature (T_m)

During the annealing step of PCR, primers and probes hybridize to targets, forming short duplexes. The stability of these duplexes is described by the melting temperature: the temperature at which an oligonucleotide duplex is 50% single-stranded and 50% double-stranded (Figure 1).

Melting temperature is a key design parameter. Inaccurate T_m predictions increase the probability of failed assay design. We provide several free software tools (SciTools programs; see Section 6) on the website that can predict T_m values from oligonucleotide sequences and reaction composition. It is often mistakenly believed that T_m is solely a property of the oligonucleotide sequence and independent of experimental conditions. Melting temperature depends on oligonucleotide sequence, oligonucleotide concentration, and cations present in the buffer, specifically monovalent ([Na^+]) and divalent ([Mg^{2+}]) salt concentrations (Figure 2). For this reason, the melting temperature for specific experimental conditions should be calculated using IDT SciTools programs (see Section 6 for information on which SciTools programs best fits your needs).

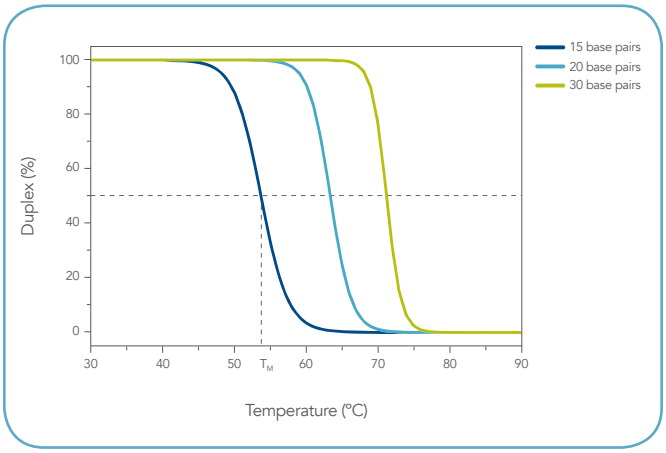


Figure 1. Melting profiles for primers of various lengths. Reactions were performed using a PCR buffer containing 1 mM Mg^{2+}, 50 mM KCl, 10 mM Tris. GC content for all primers was ~50%.

Free, online tool predicts T_m values

The OligoAnalyzer Tool has been the result of continuing thermodynamics research and innovation at IDT. It takes into account the effects of oligonucleotide, cation, dNTP, and salt concentrations, oligonucleotide sequence, and nearest-neighbor interactions. Consideration of all these factors enables accurate prediction of oligonucleotide T_m specific to your reaction conditions.

Predictive algorithms have recently been significantly improved; the nearest-neighbor method predicts T_m with a higher degree of accuracy than previously used methods [7]. Older formulas, which do not take into account interactions between neighboring base pairs, provide T_m predictions that are too inaccurate for real-time PCR design. IDT scientists have published experimental studies on the effects of Na^+, K^+, and Mg^{2+} on the stability of oligonucleotide duplexes and have proposed a model with greater accuracy [8]. The linear T_m correction has previously been used to account for salt stabilizing effects, but melting data from a large oligonucleotide set demonstrated that non-linear effects are substantial and must be considered [8].

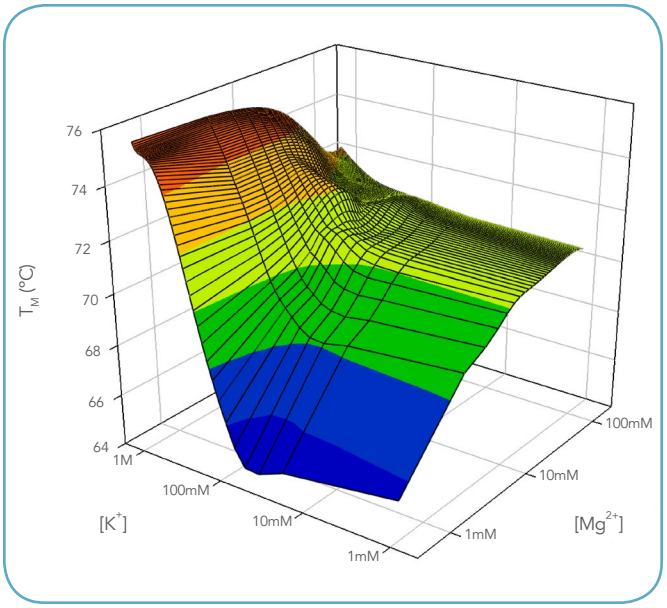


Figure 2. Salt concentration affects T_m. The stability of a 25 bp duplex (CTG GTC TGG ATC TGA GAA CTT CAG G) varies with K^+ and Mg^{2+} concentrations. Competitive binding of ions to DNA is observed.

PCR buffers also contain deoxynucleoside triphosphates (dNTPs), which bind magnesium ions (Mg^{2+}) with much higher affinity than DNA. Since dNTPs decrease the activity of free Mg^{2+}, T_m may be also decreased [8] (Figure 2). The best predictive algorithm considers this effect as well. IDT SciTools design tools (see Section 6) employ the latest nearest-neighbor method, thermodynamic parameters [9–11], and the improved salt effects model [8] to achieve state-of-the-art predictions of melting temperatures with an average error of approximately 1.5°C.

c. Reporter dyes

The choice of fluorescent dye will depend on the instrument you are using and the compatibility of the dye with the instrument. Table 2 lists dyes we recommend that are compatible with common real-time PCR instruments. However, this list is limited and may be subject to change. Refer to your instrument manufacturer's guidelines to verify compatible dyes and correct cycling conditions. FAM is the most popular of these dyes and is often more sensitive than some of the other dyes available.

Instrument	Dye and max emission wavelength																	
	518 nm	536 nm	548 nm	551 nm	556 nm	557 nm	563 nm	570 nm	580 nm	605 nm	610 nm	615 nm	615 nm	640 nm	665 nm	667 nm	670 nm	710 nm
	FAM	TET	JOE	Yakima Yellow	HEX	MAX	TYE 563	Cy3	TAMRA	ROX	LC Red 610	Texas Red	TEX 615	LC 640	TYE 665	Cy5	Quasar 670	LC 705
Agilent																		
AriaMx	•	o	o	o	•	o	o	•	o	•	X	X	X	o	o	•	X	X
Applied Biosystems																		
7000	•	o	•	o	o [1]	o	X	X	•	#	X	X	X	X	X	X	X	X
7300 (Prism)	•	X	•	o	o [1]	o	X	X	•	#	X	X	X	X	X	X	X	X
7500 (Prism)	•	X	•	o	o [1]	o	o	•	•	#	o	•	o	o	o	•	o	X
7500 HT (Fast)	•	X	•	o	o [1]	o	o	•	•	#	o	•	o	o	o	•	o	X
7700	•	•	•	o	•	o	X	X	•	#	X	X	X	X	X	X	X	X
7900 (Prism)	•	•	•	o	o [1]	o	X	X	•	#	X	X	X	X	X	X	X	X
7900HT	•	•	•	o	o [1]	o	X	X	•	#	X	X	X	X	X	X	X	X
StepOne	•	o	•	o	o [1]	o	X	X	X	#	X	X	X	X	X	X	X	X
StepOnePlus	•	o	•	o	o [1]	o	X	X	•	#	X	X	X	X	X	X	X	X
Viia 7	•	•	•	o	•	o	o	o	•	#	X	o	o	X	o	o	X	o
QuantStudio 3	•	•	•	o	•	o	o	•	•	•	X	•	o	X	X	X	X	X
QuantStudio 5	•	•	•	o	•	o	o	•	•	•	X	•	X	X	o	•	X	X
QuantStudio 6 Flex	•	o	•	o	o [1]	o	o	•	•	#	o	•	o	o	o	o	o	X
QuantStudio 7	•	o	•	o	o [1]	o	o	•	•	#	o	•	o	o	o	o	o	X
QuantStudio 12K flex	•	•	•	o	•	o	o	o	•	#	o	o	o	o	o	o	o	X
BioRad																		
CFX384	•	o	o	o	•	o	X	X	o	•	o	•	•	o	o	•	•	X
CFX96	•	o	o	o	•	o	X	X	o	•	o	•	•	o	o	•	•	X
Chromo4	•	•[1]	o	o	•	o	X	X	X	X	X	X	X	X	X	X	X	X
Connect	•	•	o [1]	o	• [1]	X	X	X	X	X	X	X	X	X	X	X	X	X
iCycler	•	o	o	o	•	o	o	o	o	o	o	•	o	o	o	•	X	X
MiniOpticon	•	o	o	o	•	o	X	X	X	X	X	X	X	X	X	X	X	X
MiniOpticon 2	•	o	o	o	•	o	X	X	X	X	X	X	X	X	X	X	X	X
MyIQ2	•	o	o	o	•	o	X	X	X	X	X	X	X	X	X	X	X	X
MyIQ5	•	o	o	o	•	o	o	o	•	o	o	•	o	o	o	•	X	X
QX100	•	X	o	o	• [1]	X	X	X	X	X	X	X	X	X	X	X	X	X
QX200	•	X	o	o	• [1]	X	X	X	X	X	X	X	X	X	X	X	X	X
Cepheid																		
Smartcycler	•	•	o	o	o	o	o	•	X	o	o	•	o	o	o	•	o	X
Smartcycler II	•	•	o	o	o	o	o	•	X	o	o	•	o	o	o	•	o	X
Illumina																		
Eco	•	o	o [1]	o	• [1]	X	X	X	X	•	X	o	o	X	o	•	•	X
Qiagen																		
Rotor-gene Q	•	•	•	o	•	•	X	X	X	•	•	•	o	X	o	•	o	•
Rotor-gene 6000	•	•	•	o	•	•	X	X	X	•	•	•	•	X	•	•	•	X
Roche																		
LC 2.0	•[2]	X	•[2]	o	•[2]	•[2]	X	X	X	•[2]	•[2]	•[2]	•[2]	•[2]	•[2]	•[2]	•[2]	•[2]
LC480	•	o	• [1]	o	• [1]	o	o	o	o	o	•	o	o	•	o	•	X	o
LC1536	•	o	o	o	•	o	o	o	o	o	o	o	o	o	o	o	X	X
LC Nano	•	o	o	o	•	o	o	o	o	o	•	•	o	o	o	•	X	X
LC96	•	X	o [1]	o	•	X	X	X	X	X	•	•	•	X	X	•	X	X

• Supplier provided or recommended reporter dyes
o Instrument capable dyes, but may require calibration
X Instrument incapable of supporting
Instrument uses channel for a reference dye

1 Instrument works with VIC, so JOE or Yakima Yellow can serve as an alternative if calibrated/tested.
2 Roche recommends running color compensation for any dye set used.

Table 2. Instrument compatibility with reporter dyes.

d. Multiplex qPCR

In multiplex qPCR, multiple targets are amplified in a single reaction tube. Each target is amplified by a different set of primers and a uniquely-labeled probe that will distinguish each PCR amplicon. Multiplexing provides some advantages over single-reaction PCR, including requiring an overall lower amount of starting material, increased throughput, lowered reagent costs, and less sample handling. However, the experimental design for multiplexing is more complicated because the amplification of each target can affect others in the same reaction. Therefore, careful consideration of design and optimization of the reactions is critical. To incorporate all necessary parameters, we strongly recommend using a design tool for primers and probes. Suitable tools can be found in Section 6.

1. Ensure that the primers and probe sets are not complementary to each other. Use BLAST to analyze the sequences and ensure they are non-complementary. For help with using the BLAST tool, see the **IDT DECODED newsletter** article, *Tips for using BLAST to locate PCR primers*.

2. Each target must be identified by a separate reporter dye. Select dyes with little or no overlap in their emission spectra (see Table 3; also, Section 6 provides information about a useful multiplex dye selection tool). Some instruments are compatible with only certain dyes (see Section 2c, above), so check the documentation for your instrument to ensure the dyes are compatible. As a general rule, it is a good idea to select FAM for any low copy transcripts because it has a strong signal. Lower signal fluorophores can then be used for the more abundant transcripts.

3. Minimize signal cross-talk by using high quenching probes, such as IDT ZEN and TAO Double-Quenched Probes (see Section 5c).

4. Optimize individual reactions, and ensure that they each have an efficiency >90%.

5. Validate the multiplex reactions by running a multiplexed reaction alongside its corresponding singleplex reactions to ensure similar performance (Figure 3). Compare the standard curves and verify that the C_q values are similar at both the high and low ends. A good multiplex should have similar curves and similar limits of detection.

6. Optimize the multiplex reactions. Limit the primers for targets expressed at a high level to a 1:1 primer-to-probe ratio. Increase the primer-to-probe ratio for targets expressed at lower levels. Increasing the amount of enzyme and dNTPs added to the reaction may be necessary—we recommend doubling the amount of these reagents. There are a few commercially available master mixes that are specially formulated for multiplexing.

Fluorophore*	Emission wavelength (nm)	Quencher
6-FAM	520	ZEN–Iowa Black FQ†
TET	539	
HEX	555	
JOE	555	
Yakima Yellow	549	
VIC‡	554	
Cy3	564	Iowa Black RQ‖
ATTO 550§	575	
NED‡	575	
TAMRA	583	
ABY‡	580	
ATTO 565§	591	
PET‡	595	
ROX	608	
Texas Red-X	617	
JUN‡	617	
ATTO 633§	657	
LIZ‡	655	
ATTO 647§	669	
Cy5	668	TAO–Iowa Black RQ¶

* Except where noted, the fluorophores in this chart are free of licensing fees and are available from IDT as Freedom Dyes.

† Probes with 6-FAM, TET, HEX, or JOE fluorophores are also available as traditional, single-quenched probes with Black-Hole Quencher®-1 (Biosearch Technologies, Inc.; additional third-party licenses required for diagnostic use).

‡ For reference only. Not available through IDT.

§ Probes with ATTO Dyes are available from IDT.

‖ Black-Hole Quencher-2 may also be used as a quencher. However, additional third-party licenses are required for diagnostic use.

¶ Cy5 is also available as a traditional, single-quenched probe with Iowa Black RQ (license free) or Black Hole Quencher-2 (additional third-party licenses required for diagnostic use).

Table 3. Emission wavelength of fluorophores and appropriate quenchers.

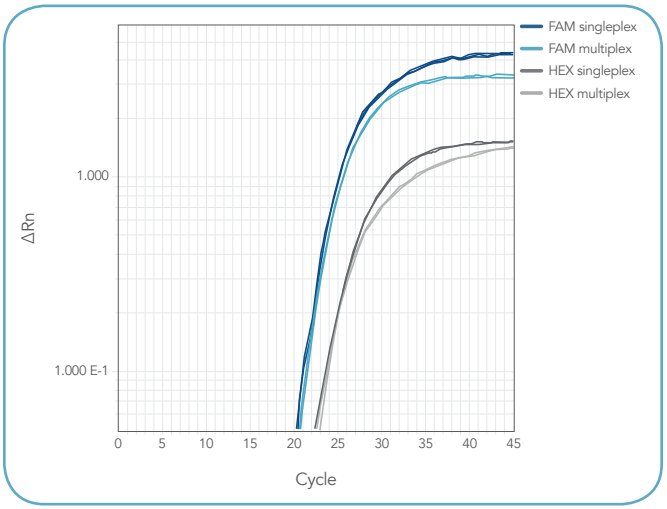


Figure 3. Validate multiplex reactions by comparing to singleplex reactions. Each reaction was performed using 50 ng cDNA with FAM or HEX dye. C_q values were 22.8 for FAM singleplex, 22.5 for FAM multiplex, and 25 for HEX singleplex and multiplex. IDT PrimeTime qPCR Assays were used to generate this data. See Section 5 for more information.

e. Predesigned assay vs. custom assay

Predesigned assays, whether from a colleague, the literature, or a commercial source, if well designed, can save a lot of research time. Why go to the trouble of designing an assay when a previously designed assay directed against the same target has been successful? Well, there are a few caveats to keep in mind. The sequence and transcript structure, as well as SNP annotation information, may have been updated since the assay was designed. Therefore, it is important to check whether the sequences you are about to use are representative of the most up-to-date sequence information. Furthermore, the assay may not have been experimentally validated and certainly was not optimized under the same research conditions—including sample type, method of sample preparation, assay reagents, and thermal cycler—you are now planning to employ. Thus, it is important to optimize and validate such assays using your own experimental setup.

Several companies offer inventoried qPCR assays that have not only been predesigned, but are also premanufactured and stocked, waiting for your order to be placed. Assays sitting in inventory were not necessarily designed with the latest target sequence information available. Genetic information is continually being updated and revised with transcripts being added, withdrawn, and reannotated. For the most accurate qPCR data, researchers must take this updated sequence information into account.

PrimeTime Predesigned qPCR Assays from IDT

IDT **PrimeTime Predesigned qPCR Assays** are unique in that they are only synthesized once an order is placed. In this way, designs can be checked against the latest sequence information.

Custom assays are especially appropriate for newly discovered and annotated transcripts, detection of microbial DNA, and custom placement of primers and probes.

3. Replicates and controls

For accurate analysis of qPCR results, each experiment needs to be set up with multiple replicates and controls (Figure 4). This section provides an explanation of each type of control, and our recommendations for setting them up and the number of replicates needed.

a. Replicates

qPCR experiments use the following two different types of replicates:

- Technical replicates (repeated RT or qPCR reactions) are used to compensate for technical noise and to increase the precision of the qPCR results. These repeated measures should not be used for statistical testing of biological hypotheses.
- Biological replicates (repeated cell cultures, or different individuals or specimens) are required to draw biologically relevant conclusions from your experiments.

The number of replicates depends on the specific needs of the experiment. For each experimental and control sample to be compared, at least 3 biological and 3 technical replicates are necessary to minimize errors in measured gene expression due to pipetting. And at least 3 biological replicates are required if you want to draw statistical conclusions. See Figure 4 for an example.

However, if you are interested in small expression differences or require higher confidence (e.g., for diagnostics), it is useful to include additional replicates. Most studies require larger numbers of samples. On the other hand, when performed by experienced users, large screening studies that include many samples and fewer replicates, may suffice.

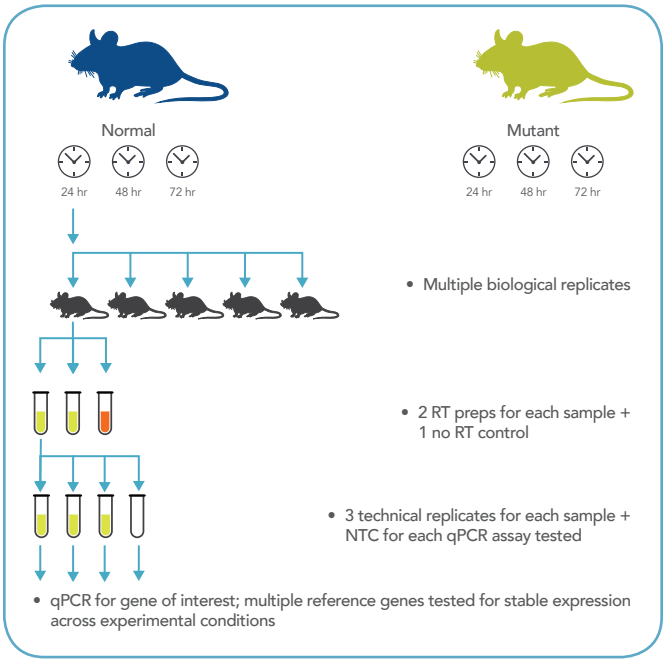


Figure 4. qPCR assay setup. Outline of the replicates and controls needed for an experiment comprising 2 different samples examined at several time points after treatment. Include 3–5 biological replicates for each time point studied. For each biological replicate studied, perform 2 reverse transcription reactions (+RT) and 1 with no RT (–RT control). For each cDNA sample generated, set up 3 technical replicates for qPCR analysis. Include a "no template control" (NTC) for each gene analyzed to identify any signal due to contamination.

b. Negative controls

We recommend the following 3 negative controls:

- The "no template control" (NTC) is an absolute requirement for all qPCR experiments. An NTC omits DNA from the PCR. This reaction serves as a general control for unwanted nucleic acid contamination or primer-dimer formation that may make the results more difficult to interpret, particularly when using SYBR® Green I dye (Life Technologies, Inc). Perform this control for each assay.
- A no reverse transcriptase control (–RT) omits the reverse transcriptase in the reverse transcription step of a RT-qPCR. The purpose of this control is to assess the amount of genomic DNA contamination present in an RNA preparation. Perform this control for any assay that might amplify genomic DNA.
- A no amplification control omits the DNA polymerase from the PCR. This reaction serves as a control for background fluorescence of the PCR assay and probe stability.

c. Positive controls

- An exogenous positive control is external DNA or RNA carrying a target of interest.

  These control reactions will alert you to any components in the sample that might inhibit reverse transcription and/or PCR [12]. IDT synthesizes gBlocks Gene Fragments, custom genes, and Ultramer DNA Oligonucleotides, which can serve as exogenous positive controls with known starting copy number.

- An endogenous positive control is a native target that is present in the experimental sample of interest and can serve as a normalizer or reference among samples.

  These control reactions will correct for quantity and quality differences among samples. We recommend that you test at least 2, but preferably 3, normalizer (reference or housekeeping) genes to ensure accurate internal controls. The most appropriate normalizing gene to use will depend on the RNA source and experimental conditions of the sample you will be testing. It is best practice to screen multiple genes under the experimental conditions employed to determine which expression levels fluctuate the least. Programs, such as qBase+ (Biogazelle), can be used to evaluate the performance of various normalizers. The most commonly used normalizers are listed in Table 4 [13]. Alternatively, review the literature for the genes tested on samples with conditions similar to yours. If you choose a normalizing gene from the literature, be sure to perform a reaction to verify that the gene expression levels do not fluctuate across samples before you use it as a control.

Gene ID	Description
18S	18S ribosomal RNA
ACTB	actin, beta
ALDOA	aldolase A, fructose-bisphosphate
ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha
B2M	beta-2-microglobulin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GUSB	glucuronidase, beta
HMBS	hydroxymethylbilane synthase
HPRT1	hypoxanthine phosphoribosyltransferase 1
HSPCB	heat shock 90kDa protein 1, beta
IPO8	importin 8
LDHA	lactate dehydrogenase A
NONO	non-POU domain containing, octamer-binding
PGK1	phosphoglycerate kinase 1
POLR2A	polymerase (RNA) II (DNA directed) polypeptide A
PPIA	peptidylprolyl isomerase A (cyclophilin A)
RPL11	ribosomal protein L11
RPL19	ribosomal protein L19
RPL32	ribosomal protein L32
RPLP0	ribosomal protein, large, P0
RPS18	ribosomal protein S18
RPS27A	ribosomal protein S27a
SFRS9	splicing factor, arginine/serine-rich 9
TBP	TATA box binding protein
TFRC	transferrin receptor
UBC	ubiquitin C
YWHAZ	3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

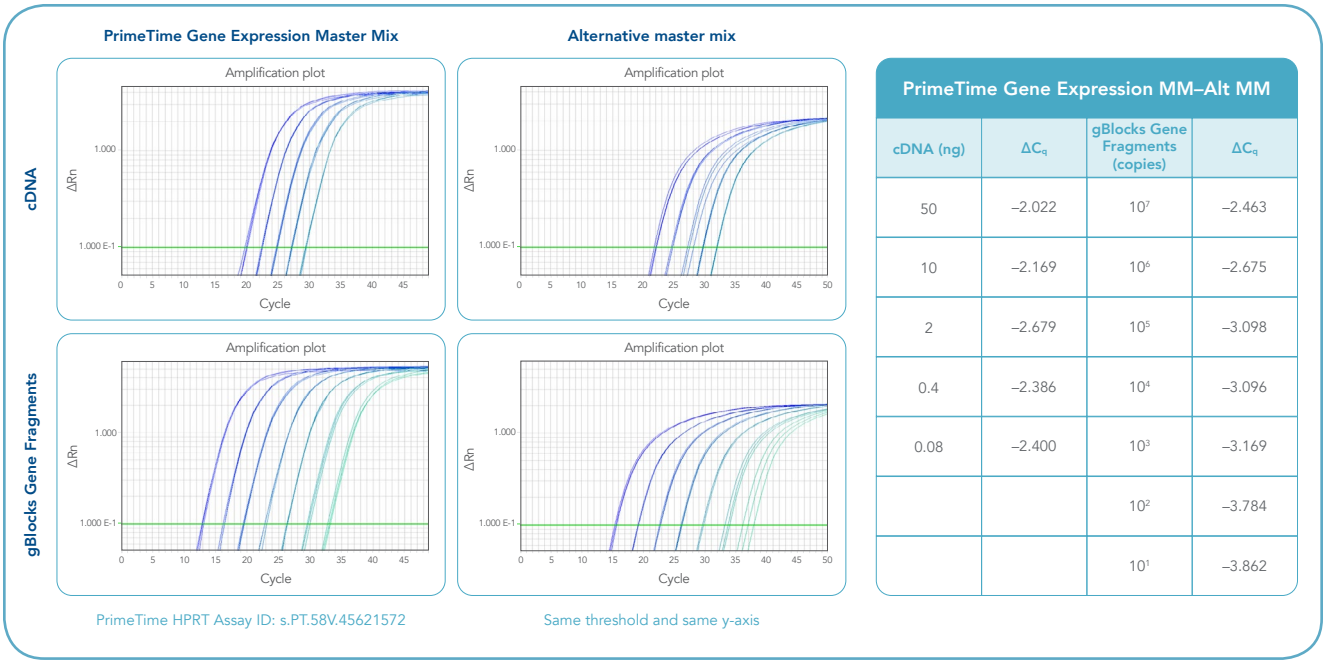
Table 4. Genes commonly used for normalization. Several of these reference gene assays are offered as PrimeTime Predesigned qPCR Assays. See Reference Gene Assays for more information.

4. Master mixes

Master mixes prepared by manual addition of components (reaction buffer, dNTPs, $MgCl_2$, and Taq polymerase) allow maximum flexibility, because components can be adjusted according to experimental needs. However, most researchers use commercial master mixes optimized to work with most standard assays. It is important to note that small changes in the master mix formulation can significantly affect assays, and some formulations are designed for use with specific cycling conditions (that is, standard or fast cycling conditions). Figure 5 provides an example of how master mix choice can affect assay efficiency and sensitivity.

A brief description of the role of the different components in a master mix is given below.

- Buffer—required to maintain optimum pH and salt conditions.
- $MgCl_2$—required to stabilize primer and probe interactions with DNA and as a cofactor for Taq polymerase. Occasionally, it may be necessary to add more $MgCl_2$ to the master mix to achieve optimum amplification results.
- dNTPs—the building blocks for DNA synthesis. Some master mixes include dUTP and UNG enzyme to eliminate carryover of product from a previous PCR. However, dUTP is not incorporated as efficiently as dTTP and might affect amplification.
- UNG (Uracil N-glycosylase) enzyme—used in some master mixes to eliminate PCR carryover contamination by degrading any PCR product with incorporated uracil bases. If a master mix includes dUTP, it is necessary to adapt cycling conditions to include an UNG step to degrade any previous PCR product.
- Polymerase—master mixes normally contain modified DNA polymerases to eliminate nonspecific priming that may occur before the initial denaturation step. Hot start polymerases have been modified—through use of an antibody, chemical modification, or aptamers—to be inactive at low temperatures. These modified enzymes require activation by heating at 95°C for 2–10 minutes during the initial denaturation step.
- ROX dye—some thermal cyclers require the use of an internal reference dye, such as ROX dye, for normalization across wells and to account for pipetting errors. Therefore, certain master mixes are available with different formulations of ROX. Refer to your instrument user manual for instructions.



PrimeTime Gene Expression MM–Alt MM			
cDNA (ng)	ΔC_q	gBlocks Gene Fragments (copies)	ΔC_q
50	–2.022	10^7	–2.463
10	–2.169	10^6	–2.675
2	–2.679	10^5	–3.098
0.4	–2.386	10^4	–3.096
0.08	–2.400	10^3	–3.169
		10^2	–3.784
		10^1	–3.862

Figure 5. Master mix composition affects assay efficiency and sensitivity. PCRs contained PrimeTime HPRT qPCR Assays (primers and probes) and either PrimeTime Gene Expression Master Mix + reference dye or another commercial master mix and separate reference dye. cDNA (upper panels) or gBlocks Gene Fragments (lower panels) were used as template. PCRs were run in triplicate on a 7900HT Real-Time PCR System (Thermo Fisher Scientific). With assays, templates, and reaction conditions kept constant, varying only the master mix, reactions using PrimeTime Gene Expression Master Mix were consistently more efficient and sensitive.

5. PrimeTime qPCR Assays and associated products

We have developed a selection of qPCR products to assess gene expression. These include predesigned probe- and primer- based assays covering transcriptome targets in human, mouse, and rat. Custom assays may be created for any sequence from any species using the PrimerQuest Tool.

You can obtain IDT qPCR reagents as ready-to-use solutions with PrimeTime probe- or primer-based assays and master mix, or create a completely custom option from our probe catalog.

a. PrimeTime Predesigned qPCR Assays

PrimeTime Predesigned qPCR Assays are available for the majority of human, mouse, and rat transcripts in the NCBI database—where primer and probe designs meet our strict selection criteria. These assays are designed using a proprietary algorithm. In addition to optimized oligo T_m (base composition, oligo length, etc.), the bioinformatic calculations account for factors, such as SNPs (based on current NCBI RefSeq releases), cross-react searches to avoid off-target amplification, recognition of splice variants, and secondary structure predictions. The IDT web-based ordering system (Section 6a) allows users to filter, sort, and select assays based on characteristics like exon location, splice variant targets, RefSeq number, or gene symbol.

It is important to note that, unlike many other commercially available inventoried assays, PrimeTime Predesigned qPCR Assays are not manufactured and stocked, but are predesigned only. In this way, updates to sequence and SNP databases can be taken into account.

Predesigned assays are guaranteed to perform with PCR efficiencies of 90–110% and R^2 >0.99. If you find an assay does not meet this criteria, it will be replaced with another at no additional charge. We also provide primer sequences with each order to assist with best practices in research reporting and reproducibility—specifically, sequence transparency [5].

Assays consist of a forward primer, a reverse primer, and a qPCR probe all delivered in a single tube. In addition, for the standard and XL sizes, dye-quencher combination and custom primer-to-probe ratio can be specified to meet unique experimental demands. Each assay is made to order with estimated shipping in 2–4 days from order receipt. Each oligonucleotide undergoes 100% QC by mass spectrometry, and all QC results are provided free of charge to you on the IDT website.

Learn more about IDT **PrimeTime Predesigned qPCR Assays**.

b. PrimeTime Custom qPCR Assays

Custom 5′ nuclease assays can also be designed for any sequence in any species using the PrimerQuest Tool (see Section 6). The tool includes optimized preset design parameters for PCR and qPCR, or customize parameters for other applications.

PrimeTime Custom qPCR Assays consist of a forward primer, a reverse primer, and a qPCR probe all delivered in a single tube. In addition, for the standard and XL sizes, dye–quencher combination and custom primer-to-probe ratio can be specified to meet unique experimental demands. Each assay is made to order with estimated shipping in 2–4 days from order receipt. Each oligonucleotide undergoes 100% QC by mass spectrometry, and all QC results are provided free of charge to you on the IDT website.

Learn more about **PrimeTime Custom qPCR Assays**.

c. ZEN and TAO Double-Quenched Probes

ZEN/Iowa Black FQ and TAO/Iowa Black FQ double-quenched probes provide superior performance compared to traditional single-quenched probes. While traditional probes have approximately 20–30 bases between the fluorophore and the quencher, the internal ZEN or TAO Quencher decreases that length to only 9 bases. This shortened distance, particularly when combined with the traditional 3′ end quencher, leads to a much more thorough quenching with much lower background and enables the use of much longer probes for designing in AT-rich target regions. In addition to the significantly decreased background, Double-Quenched Probes also provide consistently reduced C_q values and improved precision when compared to traditional probes. Use of Double-Quenched Probes can deliver both increased sensitivity and precision for your qPCR experiments.

For more information, download the **PrimeTime Custom qPCR Probes Flyer**.

Other probe modifications

LNA probes

LNA (locked nucleic acid) is a modified nucleic acid monomer. When incorporated into a probe, LNA imparts heightened structural stability to the target sequence leading to increased hybridization temperature. See **PrimeTime LNA qPCR Probes** for more information.

MGB probes

MGB Eclipse® Probes (Elitech Group) and GMP companion primers are provided by our GMP and ISO 13485-certified production group and are ideal for qPCR assays in diagnostic and clinical applications. Our range of fluorophore options (FAM, HEX, TET, and Yakima Yellow) allows you to ensure compatibility with your instrument and more easily design multiplex assays.

Freedom Dyes

Freedom Dyes are license-free, fluorescent dyes, for use in commercial or diagnostic applications. They are available for commonly used dye wavelengths and can be paired with ZEN, TAO, and Iowa Black quenchers to create qPCR probes with lower background and higher signal.

d. PrimeTime Gene Expression Master Mix

PrimeTime Gene Expression Master Mix is optimized to support probe-based qPCR assays for gene expression analysis. This master mix is guaranteed to provide assay efficiencies >90% when used with PrimeTime qPCR Assays in two-step RT-qPCR. It is also compatible with other primers and probes.

Each order includes a 2X master mix solution (antibody-mediated, hot-start DNA polymerase; dNTPs; $MgCl_2$; enhancers; and stabilizers) and a separate reference dye stock solution.

PrimeTime Gene Expression Master Mix is shipped at ambient temperature. Elimination of shipping on dry ice saves your research money, minimizes shipping delays, and benefits the environment.

To view our extensive testing that shows ambient shipping does not impact the function of the master mix, see our white paper.

Learn more about PrimeTime Gene Expression Master Mix.

e. Other useful reagents from IDT

i. Ultramer DNA Oligonucleotides

IDT synthesis systems and chemistries allow high fidelity synthesis of very long single-stranded oligonucleotides (up to 200 bases). Ultramer Oligonucleotides are suitable for demanding applications such as cloning, shRNA, mutagenesis, and gene construction. Ultramer Oligonucleotides serve as excellent controls and can be used as standards of known concentration.

Learn more about Ultramer DNA Oligonucleotides.

ii. gBlocks Gene Fragments

Up to 3000 bp in length, these double-stranded DNA fragments are constructed using Ultramer Oligonucleotides and are sequence-verified. gBlocks fragments serve as excellent controls and can be used as standards of known concentration. See the IDT Technical report, *gBlocks Gene Fragments as qPCR standards*, for details. By acquiring entire target fragments as gBlocks Gene Fragments, researchers can save a great amount of time and effort.

Learn more about gBlocks Gene Fragments.

iii. Gene sets

We have compiled suggested gene lists for targeting some commonly studied pathways in human, mouse, and rat. These include housekeeping, apoptosis, cytokines, growth factor, and tumor metastasis genes. Gene lists for additional human signaling pathways (Notch, TLR, WNT, etc.), biochemical pathways, and genes that have been associated with certain cancers, are also available as an Extended Gene Set List. View these lists here.

iv. Reference gene assays for normalization

We provide a selection of predesigned assays for common normalization (housekeeping) genes for human, mouse, and rat. Review the details of these under the Reference Gene Assays section.

v. Nuclease detection and control reagents

We also offer several reagents for rapid detection and elimination of RNases and DNases. The detection assays, RNaseAlert® Kit (Life Technologies, Inc) and DNaseAlert Kit, are effective for testing lab reagents, equipment, and other lab supplies. Results can be read visually for qualitative assessment of contamination or quantified using fluorometry. A Nuclease Decontamination Solution that irreversibly inactivates nucleases and that can be applied to most laboratory surfaces is also available.

Learn more about IDT nuclease detection and control reagents.

vi. IDTE Buffer

We provide 1X TE Buffer (10 mM Tris, 0.1 mM EDTA; available at pH 7.5 or 8.0) for initial resuspension and storage of DNA oligonucleotides. DNA oligonucleotides can be damaged by prolonged incubation or storage in even mildly acidic solutions; DNA dissolved in distilled water often has a final pH <5.0 and is at risk of depurination. IDTE is guaranteed to be nuclease-free. Each lot is tested using our RNaseAlert and DNaseAlert Kits to document the absence of nuclease activity.

Learn more about IDTE Buffer and other reagents available from IDT.

vii. RNase-free H_2O

Nuclease-Free Water is convenient for quick dilutions of storage stocks, or for especially short-term storage. For more information on various storage media and their effects on oligo stability, click here.

6. Software tools for assay design and primer evaluation

a. Predesigned qPCR Assay selection tool

The **IDT Predesigned qPCR Assay selection tool** is a dedicated design tool for the library of PrimeTime Predesigned qPCR Assays. If your target is a human, mouse, or rat sequence, this program offers the highest level of bioinformatics analysis, including BLAST search to avoid cross reaction and off-target amplification, and recognition of splice variants.

b. PrimerQuest Tool

The **PrimerQuest design tool** is highly customizable and useful for the design of qPCR assays with non-standard requirements. For example, you can use this design tool to direct the assay towards specific regions of your target, or you can specify primer or probe sequences. Use the PrimeQuest Tool to adjust reaction conditions, add a probe to a set of previously designed primers, define primer positions, and include or exclude sequences from the assay designs. So if your design requires more demanding customization, this highly flexible program can be of great use. IDT Technical Support is available to offer assistance with this program to help you meet your specific design challenges. Contact them at applicationsupport@idtdna.com.

c. PrimeTime Multiplex Dye Selection Tool

The **PrimeTime Multiplex Dye Selection Tool** helps you select dye combinations that are compatible with your qPCR instrument. Multiplexing 2 to 5 qPCR targets in a single reaction can save time and money.

d. NCBI BLAST tool

NCBI's Basic Local Alignment Search Tool (**BLAST tool**) is an incredibly powerful tool that can be used to efficiently query the massive GenBank database to find regions of local similarity between sequences. It calculates the statistical significance of matches and can be used to select primers and probe sequences for qPCR assays. For more information, see the **IDT DECODED newsletter** article, *Tips for using BLAST to locate PCR primers*.

e. Additional tools and calculators

In addition to the assay design tools described in Sections 6a–c, SciTools programs include calculators and a renown oligonucleotide analysis tool that may be helpful for setting up RT-qPCR experiments, especially when working with custom assays.

i. OligoAnalyzer Tool is the most heavily used IDT SciTools program. This tool analyzes the properties of oligonucleotide sequences. By simply inputting your sequence, you can find out its length, GC content, melting temperature range, molecular weight, extinction coefficient, and optical density. The program also provides information about secondary structures, such as hairpin and primer-dimer formation, as well as mismatches, effects of modifications or buffer conditions on those properties, and an assortment of other useful information that can affect RT-qPCR or other application performance.

ii. The Dilution Calculator is an easy-to-use calculator designed to compute the volume of concentrated oligonucleotide stock required to achieve a desired dilution volume and concentration.

iii. The Resuspension Calculator determines the volume of buffer or water to add to a dry or lyophilized oligonucleotide to reach a desired final concentration.

7. Summary

You should now be familiar with the critical variables in qPCR assay design, setup, and optimization. Attention to these will ensure that your qPCR experiments provide quantitative, accurate, and interpretable data. Reporting your optimized conditions for these variables has become a necessity for publication of experimental data and will enable other researchers to replicate your findings, a pillar of the MIQE guidelines [3–5].

We have devoted significant research resources towards improving qPCR assay performance and reproducibility both for our customers and our own researchers. As we do so, we have documented what we have learned in educational articles and videos. Visit us at www.idtdna.com for more information.

8. References

1. Bustin S, Huggett J. (2017) qPCR primer design revisited. Biomol Detect Quant, 14:19–28.
2. The International HapMap Consortium. (2007) A second generation human haplotype map of over 3.1 million SNPs. Nature, 449:851–861.
3. Bustin SA, Benes V, et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem, 55(4):611–622.
4. Bustin SA, Beaulieu J-F, et al. (2010) MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. BMC Mol Biol, 11:74.
5. Bustin SA, Benes V, et al. (2011) Primer sequence disclosure: a clarification of the MIQE guidelines. Clin Chem, 57(6):919–921.
6. Poon K, Macgregor RB Jr. (1998) Unusual behavior exhibited by multi-stranded, guanine-rich DNA complexes. Biopolymers, 45(6):427–434.
7. Owczarzy R, Vallone PM, et al. (1997) Predicting sequence-dependent melting stability of short duplex DNA oligomers. Biopolymers, 44(3):217–239.
8. Owczarzy R, Moreira BG, et al. (2008) Predicting stability of DNA duplexes in solutions containing magnesium and monovalent cations. Biochem, 47(19):5336–5353.
9. SantaLucia J Jr, Hicks D. (2004) The thermodynamics of DNA structural motifs. Annu Rev Biophys Biomol Struct, 33:415–440.
10. McTigue, PM, Peterson RJ, Kahn JD. (2004) Sequence-dependent thermodynamic parameters for locked nucleic acid (LNA)-DNA duplex formation. Biochemistry, 43(18):5388–5405.
11. Xia T, SantaLucia Jr J, et al. (1998) Thermodynamic parameters for an expanded nearest neighbor model for formation of RNA duplexes with Watson-Crick base pairs. Biochemistry, 37(42):14719–14735.
12. Nolan T, Hands RE, et al. (2006). SPUD: a quantitative PCR assay for the detection of inhibitors in nucleic acid preparations. Anal Biochem, 351(2):308–310.
13. Eisenberg E, Levanon EY. (2003) Human housekeeping genes are compact. Trends Genet, 19:362–365.

Real-time qPCR assay design guide

Guidance for gene expression experiments

First edition

Technical support:

applicationsupport@idtdna.com