Thermo SCIENTIFIC

PRODUCT INFORMATION

Thermo Scientific
DyNAmo Flash Probe qPCR Kit
DyNAmo ColorFlash Probe qPCR Kit

#F-455S

Lot 00000000 Expiry Date ____

Store at -20°C in the dark

www.thermoscientific.com/onebio

Contents

COMPONENTS OF THE KIT

DyNAmo Flash Probe qPCR Kit	**F-455S**	**F-455L**	**F-455XL**
2X master mix (contains hot-start *Tbr* DNA polymerase, optimized PCR buffer, $MgCl_2$, dNTP mix including dUTP)	1 × 1 mL (sufficient for 100 reactions of 20 µL or 40 reactions of 50 µL)	5 × 1 mL (sufficient for 500 reactions of 20 µL or 200 reactions of 50 µL)	25 × 1 mL (sufficient for 2500 reactions of 20 µL or 1000 reactions of 50 µL)
50X ROX passive reference dye (contains 25 µM ROX)	1 × 50 µL	1 × 250 µL	1 × 1.25 mL
DyNAmo ColorFlash Probe qPCR Kit	**F-456S**	**F-456L**	**F-456XL**
2X master mix with blue dye (contains hot-start *Tbr* DNA polymerase, optimized PCR buffer, $MgCl_2$, dNTP mix including dUTP)	1 × 1 mL (sufficient for 100 reactions of 20 µL or 40 reactions of 50 µL)	5 × 1 mL (sufficient for 500 reactions of 20 µL or 200 reactions of 50 µL)	25 × 1 mL (sufficient for 2500 reactions of 20 µL or 1000 reactions of 50 µL)
40X sample buffer solution with yellow dye	200 µL	1000 µL	5 × 1 mL
50X ROX passive reference dye (contains 25 µM ROX)	1 × 50 µL	1 × 250 µL	1 × 1.25 mL

STORAGE

The Thermo Scientific™ DyNAmo™ Flash Probe qPCR Kit and Thermo Scientific™ DyNAmo™ ColorFlash Probe qPCR Kit are shipped on dry ice. Upon arrival, store all kit components at -20°C with minimal exposure to light. When using the 2X master mix, the leftover thawed mix can be refrozen and stored at -20°C without affecting the performance of the kit.

The yellow sample buffer solution in the DyNAmo ColorFlash Probe qPCR Kit is stable and can be stored at +4°C, but storage at -20°C with the other kit components is recommended.

DESCRIPTION

DyNAmo Flash Probe qPCR Kit (F-455) and DyNAmo ColorFlash Probe qPCR Kit (F-456) are designed for fast quantitative, real-time analysis of DNA samples from various sources using probe-based detection. Quantitative PCR (qPCR) is a useful technique for the investigation of gene expression, viral load, pathogen detection, and numerous other applications. Fast protocols provided by the DyNAmo Flash products increase instrument throughput by allowing

more runs to be performed in the same amount of time. Fast assays are especially important when sample processing, qPCR run and data analysis need to be accomplished during the same day.

The performance of the DyNAmo Flash Probe qPCR Kits is based on a hot-start *Thermus brockianus (Tbr)* DNA polymerase. *Tbr* DNA polymerase is chemically engineered to be inactive at room temperature. The inactivation prevents the extension of nonspecifically bound primers during reaction setup and therefore increases PCR specificity. Reactions can be set up at room temperature. The initial denaturation step in the PCR protocol reactivates the polymerase (hot start). The buffer composition of the DyNAmo Flash Probe qPCR master mixes is specifically optimized for shorter annealing and extension times without compromising the qPCR performance.

The qPCR master mix in DyNAmo ColorFlash Probe qPCR Kit (F-456) contains a blue dye that helps to visualize reaction mixes during setup. Additionally, a sample buffer with a yellow dye is provided to keep track of pipetting. Using the yellow sample buffer is optional, but it significantly helps to visualize the wells where sample has already been added. The PCR premix without the sample is blue, and adding the sample turns the reaction mix green.

The reaction chemistry of DyNAmo Flash Probe qPCR Kits is applicable to most block-based and capillary-based real-time PCR instruments, including those from Applied Biosystems, Roche, Bio-Rad Laboratories, Corbett Research, and Stratagene. When RNA is used as the starting material, we recommend Thermo Scientific™ Maxima™ First Strand cDNA Synhesis Kit for RT-qPCR (K1641) or Thermo Scientific™ DyNAmo™ cDNA Synthesis Kit (F-470) for producing cDNA in order to ensure high-quality results. For SYBR Green based detection, we recommend Thermo Scientific™ DyNAmo™ Flash or ColorFlash SYBR Green qPCR Kits (F-415 and F-416).

4. NOTES ABOUT REACTION COMPONENTS

Table 1. General recommendations.

Categories	**Comments**
Kit storage	Store at -20°C
Consumables	Follow the recommendations of the PCR instrument manufacturer
Reaction volume	20-50 µL
Amplicon size	< 250 bp
Template amount	Depends on template type and quality. In general, do not use more than 200 ng of genomic DNA in a 20 µL reaction.
Primer and probe design	Use primers with matched Tm. Avoid inter-primer and intra-primer complementary sequences. We recommend calculating Tm by the nearest-neighbor method as described by Breslauer *et al.* (1986) *Proc. Nat. Acad. Sci.* 83: 3746–50. Instructions for Tm calculation and a link to a calculator using the nearest-neighbor method can be found on the Thermo Scientific website (www.thermoscientific.com/pcrwebtools).

4.1. qPCR master mix

The 2X qPCR master mix contains hot-start *Tbr* DNA polymerase, optimized PCR buffer, $MgCl_2$ and dNTP mix including dUTP. Only the template, primers and probe need to be added by the user. The master mix in DyNAmo Flash Probe qPCR Kit (F-455) is colorless, but the master mix in DyNAmo ColorFlash Probe qPCR Kit (F-456) contains a blue dye that helps

keeping track of pipetting of the master mix into the reaction wells. It is easy to see which wells on a PCR plate are empty and which ones already contain the blue master mix. The absorption maximum of the blue dye is at 615 nm.

4.2. Hot start *Tbr* DNA polymerase

The 2X qPCR master mix includes a hot start *Tbr* DNA polymerase, which is a chemically reversibly inactivated enzyme. The inactivation prevents the extension of nonspecifically bound primers during reaction setup and the first heating cycle, and therefore increases PCR specificity. The initial denaturation step in the PCR protocol reactivates the polymerase (hot start). Due to the hot start polymerase, the reaction setup can be performed at room temperature. The hot start *Tbr* DNA polymerase has 5'→3' exonuclease activity, which is required for hydrolysis probe chemistries, e.g. for TaqMan® chemistry.

4.3. Probe-based detection chemistries

Many qPCR chemistries based on the use of labeled probes have been developed. Usually the probe is labeled with a fluorophore, and fluorescence of the probe is changed as a consequence of its annealing to the target DNA.

Fluorophores

The fluorophores used in real-time qPCR assays absorb light energy at one wavelength and almost immediately re-emit light energy at another, longer wavelength. Each fluorophore has a distinctive range of wavelengths at which it absorbs light and another at which it emits light. Quenchers are molecules that can accept energy from fluorophores and then dissipate it in another wavelength or without light emission. This transfer of energy between two molecules is termed FRET (Fluorescence Resonance Energy Transfer). The emission spectrum of the fluorophore and the absorption spectrum of the quencher have to overlap to accomplish optimal quenching.
Excitation and emission spectra of most commonly used fluorophores and quenchers reside in visible range 495–670 nm. The blue dye in the master mix and the yellow dye in the sample buffer of DyNAmo ColorFlash Probe qPCR Kit (F-456) absorb at 413 and 615 nm. Typically, the excitation or emission of common qPCR fluorophores is not at these wavelengths. However, dyes that have exicitation or emission close to these wavelengths, suchs as ROX and Texas Red, can appear less intensive than in reactions without the colored components, but this does not affect the specificity or sensitivity of qPCR assays.

Hydrolysis probes (TaqMan, Double Dye, etc.)

Hydrolysis probe chemistry is the most widely used probe-based chemistry in real-time PCR. A hydrolysis probe consists of a target-specific sequence, which is usually around 20 bp long. The probe has a fluorescent reporter molecule (fluorophore) in one end and a quencher in the other end of the probe. The quencher receives the energy from the fluorophore and quenches the fluorescence. During the PCR protocol the probe hybridizes to its complementary sequence in the target and one of the PCR primers anneals in the same strand close upstream from the probe. When the polymerase extends the primer it encounters the probe, hydrolyses it from the 5' end, and thus cleaves the reporter from the probe. When the reporter is cleaved it is no longer quenched and the increase in the fluorescence can be measured with the real-time PCR instrument.

4.4. PCR primers and probes

Careful primer and probe design is important to minimize nonspecific primer annealing and primer-dimer formation. Standard precautions must be taken to avoid primer-dimer or hairpin loop formation. Most primer design software tools will yield well-designed primers for use in qPCR.

With TaqMan chemistry primers and probes are usually annealed and extended at 60°C. Primers and probes should be designed so that primer Tm's are approximately 5°C above and probe Tm approximately 10°C above the annealing temperature.

The optimal concentration for the primers is usually between 0.05 and 1 µM and for the probe between 0.05 and 1 µM, but the optimum depends on the chemistry and other assay variables. Requirements for probe design and probe concentration depend on the chemistry used. For TaqMan chemistry, for example, the recommended starting concentration for the primers is 0.5 µM and for the probe 0.25 µM.

4.5 Template preparation and quality

Purity of nucleic acid templates is particularly important for qPCR, as contaminants may interfere with fluorescence detection. Most commercial DNA purification kits give satisfactory results for qPCR.

4.6 Standards

Standard curve is needed for absolute quantification and for analyzing the efficiency of the qPCR reaction (see Section 6.1). Correlation coefficient (R^2) of the standard curve indicates how well the standard curve fits the measured data and therefore reflects the reliability of the assay.

The absolute amount of the target nucleic acid (expressed as a copy number or concentration) is determined by comparison of Cq values to external standards containing a known amount of DNA. (Cq = quantification cycle, the fractional PCR cycle at which the target is quantified in a given sample. The level of Cq is set manually or calculated automatically.) The external standards should contain the same or nearly the same DNA sequence as the template of interest. It is especially important that the primer and probe binding sites are identical to ensure equivalent amplification efficiencies of both standard and target molecules.

4.7 ROX passive reference dye

For most real-time instruments ROX passive reference dye is not required, but on some instruments it is used to normalize for non-PCR-related fluorescence signal variation. Passive reference dye does not take part in the PCR reaction and its fluorescence remains constant during the PCR reaction. The amount of ROX passive reference dye needed can vary depending on the type of excitation. The amount of ROX dye needed with real-time cyclers which use argon laser as the excitation light source or which have excitation filters that are not optimal for ROX dye may be greater than with instruments that excite efficiently near 585 nm. The ROX dye is provided as a 50X solution containing 25 µM ROX in a buffer that is compatible with the qPCR reaction buffer. The optimal ROX dye concentration is usually 0.3–1X (see Table 2 for instrument-specific recommendations). Note that the use of ROX passive reference dye may not be possible with some fluorescent dyes.

With DyNAmo ColorFlash Probe qPCR Kit (F-456) the fluorescence intensity of ROX can be lower than with DyNAmo Flash Probe qPCR Kit (F-455), but this does not affect qPCR assay specificity or sensitivity. Usually the ROX concentrations recommended in Table 2 are

sufficient for passive reference dye normalization with all instruments, but it is important to make sure that the intensity is strong enough to produce stable signal for normalization.

Table 2. ROX concentration.

Real-time PCR instrument	Recommended ROX concentration
Applied Biosystems StepOne™ Real-Time PCR System	1X
Applied Biosystems 7000, 7300, 7700 Real-Time PCR Systems	1X
Applied Biosystems 7900HT Real-Time PCR System	1X
Applied Biosystems ViiA 7 Real-Time PCR System	0.3X
Applied Biosystems 7500 Real-Time PCR System	0.3X
Agilent Mx3000P® QPCR System	0.3X (optional)
Agilent Mx3005P® QPCR System	0.3X (optional)
Agilent Mx4000® QPCR System	0.3X (optional)

4.8 40X Sample Buffer with yellow dye (F-456 only)

The 40X Sample Buffer with yellow dye is included in DyNAmo ColorFlash Probe qPCR Kit (F-456). It is used to track pipetting of samples to the qPCR reactions. When using the blue master mix of the DyNAmo ColorFlash Probe qPCR Kit the PCR reaction mix is blue before sample addition. After adding the sample the reaction mix turns green, making it easy to follow pipetting of the samples.
The yellow sample buffer can be added to existing samples, and the samples can then be stored at -20°C if not used immediately. The buffer is provided as a 40X concentrate and used in 1X concentration in the final reaction. Using the yellow sample buffer is optional. The absorption maximum of the yellow dye is at 413 nm.

4.9 UDG (UNG) treatment

Due to the high sensitivity of qPCR, even minute amounts of contaminating DNA can lead to false positive results. If dUTP is used in all qPCR reactions, the carry-over contamination from previous PCR runs can be prevented by treating the reaction samples with UDG before PCR. UDG (uracil-DNA glycosylase) digests dU-containing DNA, and the digested DNA cannot act as a template in qPCR (Longo M.C. *et al.* (1990) *Gene* 93: 125–28). UDG is inactivated during the first denaturation step in PCR. The UDG treatment step (50°C for 2 min) has no negative effect on qPCR performance because the hot-start *Tbr* DNA polymerase is not reactivated at 50°C. All Thermo Scientific DyNAmo qPCR Kits contain dUTP and therefore UDG treatment can be used. We recommend using our Thermo Scientific™ Uracil-DNA Glycosylase (EN0361).

To minimize contamination risk in general, tubes or plates containing reaction products should not be opened or analyzed by gel electrophoresis in the same laboratory area that is used to set up reactions.

4.10 Reaction volume

A reaction volume from 20 to 50 µL is recommended for most real-time instruments. The minimum reaction volume depends on the real-time instrument and consumables (follow the supplier's recommendations). The reaction volume can be increased if a high template amount is used.

4.11 Multiplex qPCR

DyNAmo Flash Probe qPCR Kits can also be used in multiplex qPCR, i.e. detection of multiple targets in the same qPCR reaction. In multiplex qPCR primers and probes are specifically designed for each target, and each probe is labeled with different fluorescent label. Multiplex assays need to be carefully designed and the relative amounts of different target molecules can strongly affect the performance of the assay.

4.12 Quantification of RNA

To determine the quantity of mRNA, a reverse transcription (RT) reaction must be performed before qPCR. Thermo Scientific offers Maxima First Strand cDNA Synthesis Kit for RT-qPCR (K1641) and DyNAmo cDNA Synthesis Kit (F-470) for quantitative reverse transcription.
For additional information about the reverse transcription step, see Appendix I: cDNA synthesis.

5. REACTION SETUP AND CYCLING PROTOCOLS

- Perform the reaction setup in an area separate from nucleic acid preparation and PCR product analysis.
- As the hot-start *Tbr* DNA polymerase is inactive during PCR setup, it is not necessary to do the setup on ice.
- Make sure all the reaction components are properly mixed.
- Pipette with sterile filter tips.
- Minimize the exposure to light after adding ROX passive reference dye and/or probe to the 2X master mix.
- Minimize pipetting errors by using calibrated pipettes and by preparing premixes to avoid pipetting very small volumes.
- Use optically clear caps or sealers to achieve maximum signal.
- Use a cap sealing tool or firm finger pressure to close caps properly, or use a film sealer.
- Avoid touching the optical surface of the cap or sealing film without gloves, as fingerprints may interfere with fluorescence measurements.
- Use powder-free gloves.
- Plates or strips should be centrifuged before starting the cycling program to force the solution to the bottom of the tubes and to remove any bubbles.
- Use molecular biology grade H_2O.

5.1. General protocol for all instruments

If you are using an Applied Biosystems real-time PCR instrument, see Section 5.2.

Reaction setup

Step 3 (adding sample buffer) is optional. You can perform it if you are using DyNAmo ColorFlash Probe qPCR Kit (F-456) and wish to track pipetting when adding samples to the reactions.

1. Program the cycler as outlined in Table 4.
2. Thaw the template DNA, primers, probe and master mix (and the ROX passive reference dye, if necessary). Mix the individual solutions to ensure homogeneity. This is especially important for the master mix.
3. If using the yellow sample buffer (optional), add buffer to the samples to a concentration that will yield 1X in the final reaction volume. For example, if 5 µL of sample is to be used in a 20 µL reaction volume, 4X buffer concentration in the sample results in 1X buffer concentration in the final reaction.
4. Prepare a PCR premix by mixing the master mix, primers, probe, (ROX if used,) and H_2O. Mix the PCR premix thoroughly to ensure homogeneity. Dispense appropriate volumes into strip tubes or plate wells.
5. Add template DNA (<200 ng per 20 µL reaction) to the strip tubes or plate wells containing the PCR premix. For two-step qRT-PCR, the volume of the cDNA added (from the RT reaction) as the template should not exceed 10% of the final PCR volume.
6. Seal the strips or plate with appropriate sealer, place them in the thermal cycler and start the cycling program.

Table 3. Reaction setup for Hydrolysis probes (TaqMan, Double Dye, etc.).

Components (In order of addition)	**20 µL reaction**	**50 µL reaction**	**Final concentration**	**Comments**
2X Master mix	10 µL	25 µL	1X	Mix thoroughly. Avoid air bubble formation.
Primer mix (in H_2O)	X µL	X µL	0.5 µM fwd 0.5 µM rev	Titrate from 0.05 to 1 µM if necessary.
Probe	X µL	X µL	0.25 µM (TaqMan probe)	Titrate from 0.05 to 0.5 µM if necessary
50X ROX reference dye	(0.12–0.4 µL	(0.3–1 µL)	0.3–1X	Optional (see Section 4.7 and 5.2).
Template DNA (including yellow sample buffer)	X µL	X µL		In general, max 200 ng/20 µL reaction. Using the yellow sample buffer is optional.

For different volumes, adjust all components proportionally.

Cycling protocol

Table 4. Cycling protocol for Hydrolysis probes (TaqMan, Double Dye, etc.).

Step	Purpose	Temp	Time	Comments
	UDG incubation			Optional, see below.
1	Initial denaturation	95°C	7 min	This step is needed to activate the hot start DNA polymerase and to denature the template DNA.
2	Denaturation	95°C	5 s	
3*	Annealing/ Extension	60°C	15-30 s	In some assays, 30 s incubation time may improve the results**.
4	Data acquisition			Fluorescence data collection

* Use the Tm calculator at www.thermoscientific.com/pcrwebtools to determine Tm of the primers.Use 50 mM KCl and 0.5 µM primer concentration when calculating Tm (or the primer concentration in your reaction if optimized to other than 0.5 µM). Design primers to anneal efficiently at 60°C (Tm should be about 65°C).

** With fast ramping instruments and if high molecular weight genomic DNA is used as a template, 30 s incubation time may improve the results.

UDG incubation (optional)

If UDG enzyme is used, incubate 2 min at 50°C. This step does not negatively affect qPCR performance because the hot-start DNA polymerase is not active at 50°C. If heat-labile UDG is used, decrease the incubation temperature and increase time in accordance with the manufacturers' instructions.

Initial denaturation / reactivation

Initial denaturation at 95°C for 7 min is needed to ensure a complete reactivation of the hot-start DNA polymerase and denaturation of the template.

Denaturation

Denaturation at 95°C for 5 s is sufficient in most cases.

Annealing and extension

For most amplicons, a combined annealing and extension for 15 seconds at 60°C works well if the primers are designed to anneal efficiently at 60°C (Tm should be about 65°C). An annealing temperature of 60°C has proven to be successful for a wide range of primer pairs. With some fast ramping instruments, the annealing/extension time should be increased up to 30 s to allow complete amplification in every cycle. When genomic DNA is used as a template, an extension time of 30 seconds is recommended.
These guidelines are based on Tm values (50 mM salt and 0.5 µM primer) calculated by the nearest-neighbor method as described by Breslauer *et al.* (1986) *Proc. Nat. Acad. Sci.* 83: 3746–50. Instructions for Tm calculation and a link to a calculator using a modified nearestneighbor method can be found on the Thermo Scientific website (www.thermoscientific.com/pcrwebtools). Different software may give different Tm values.

If primer-dimers are observed, the easiest solution is often to redesign primers. Another alternative is to optimize the annealing temperature by performing additional runs, varying the annealing temperature in each by 2°C. A temperature gradient feature on the thermocycler can also be used, if available.

Number of cycles

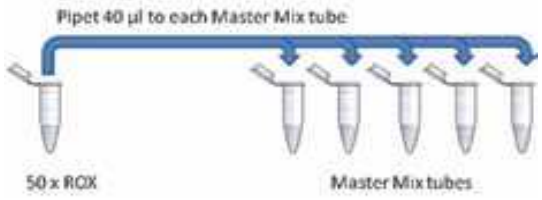
For most applications, 40 cycles of amplification should be sufficient even when the template is present at a very low copy number.

5.2 Protocol for Applied Biosystems real-time PCR instruments requiring ROX

Addition of ROX passive reference dye

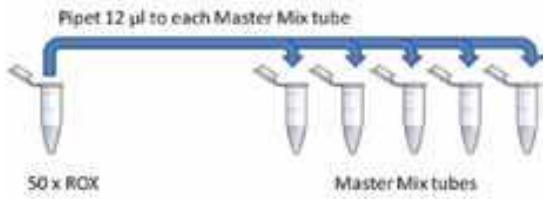
ABI 7000, 7300, 7700, 7900 and StepOne: 1X ROX final concentration

1. Thaw and carefully mix 50X ROX and 2X master mix tubes.
2. Add 40 µL of 50X ROX to each 1 mL 2X master mix tube.
3. Mix again carefully.
4. Store at -20°C.



ABI 7500, Viia7: 0.3x ROX final concentration

1. Thaw and carefully mix 50X ROX and 2X master mix tubes.
2. Add 12 µL of 50X ROX to each 1 mL 2X master mix tube.
3. Mix again carefully.
4. Store at -20°C.



Reaction setup for all Applied Biosystems models

Step 3 (adding sample buffer) is optional. You can perform it if you are using DyNAmo ColorFlash Probe qPCR Kit (F-456) and wish to track pipetting when adding samples to the reactions.

1. Program the cycler as outlined in Table 6.
2. Thaw template DNA, primers, probe(s) and 2X Master mix (where ROX passive reference dye has been added). Mix the individual solutions to assure homogeneity. This is especially important for the Master mix.
3. If using the yellow sample buffer (optional), add buffer to the samples to a concentration that will yield 1X in the final reaction volume. For example, if 5 µL of sample to be be used in a 20 µL reaction volume, 4X dye concentration in the sample results in 1X dye concentration in the final reaction.
4. Prepare a PCR premix by mixing 2X Master mix, primers, probe(s) and H_2O. Mix the PCR premix thoroughly to assure homogeneity. Dispense appropriate volumes into strip tubes or plate wells. Use reverse pipeting technique to avoid bubbles.
5. Add template DNA (< 200 ng/20 µL reaction) to the strip tubes or plate wells containing the PCR premix. For two-step qRT-PCR, the volume of the cDNA added (from the RT reaction) should not exceed 10% of the final PCR volume.
6. Seal the strips or plate with appropriate sealer, place them in the thermal cycler and start the cycling program.

Table 5. Reaction setup for Applied Biosystems real-time PCR instruments.

Components (In order of adittion)	**20 µL reaction**	**50 µL reaction**	**Final concentration**	**Comments**
2X Master mix with ROX added (see instructions above)	10 µL	25 µL	1X	Mix thoroughly. Avoid air bubble formation.
Primer mix (in H_2O)	X µL	X µL	0.5 µM fwd 0.5 µM rev	Titrate from 0.05 to 1 µM, if necessary
Probe	X µL	X µL	0.25 µM (TaqMan probe)	Titrate from 0.05 to 1 µM, if necessary
Template DNA (including yellow sample buffer)	X µL	X µL		In general, max 200 ng/20 µL reaction. Using the yellow sample buffer is optional.
H_2O	add to 20 µL	add to 50 µL		

Cycling protocol for all Applied Biosystems models

Table 6. Cycling protocol for Applied Biosystems real-time PCR instruments.

Step	**Temp.**	**Time**	**Cycles**
Initial denaturation	95°C	7 min	1
Denaturation	95°C	5 s	40
Annealing/Extension	60°C	30 s*	

* A shorter annealing/extension step (down to 15 s) can be used with the following instruments: ABI 7000, 7700 and 7900.

6. ANALYSIS

6.1. Absolute quantification

Absolute quantification is performed by plotting samples of unknown concentration on a standard curve generated from a dilution series of template DNA of known concentration. Typically, the standard curve is a plot of the quantification cycle (Cq) against the logarithm of the amount of DNA. A linear regression analysis of the standard plot is used to calculate the amount of DNA in unknown samples. The slope of the equation is related to the efficiency of the PCR reaction. The PCR efficiency should be the same for standards and samples for quantification to be accurate. The PCR efficiency of the samples can be determined by doing a dilution series of these samples.

For a graph where Cq is on the y axis and log(RNA copy #) on the x axis:
PCR efficiency = $((10^{-1/slope}) - 1) \times 100\%$
A slope of –3.322 corresponds to 100% efficiency.
For a graph where log(RNA copy#) is on the y axis and Cq on the x axis:
PCR efficiency = $((10^{-1/slope}) - 1) \times 100\%$
A slope of –0.301 corresponds to 100% efficiency.

6.2. Relative quantification

Relative quantification is used to determine the ratio between the quantity of a target molecule in a sample and in the calibrator (healthy tissue or untreated cells, for example). The most common application of this method is the analysis of gene expression, such as comparisons of gene expression levels in different samples, for example. The target molecule quantity is usually normalized with a reference gene (see chapter 'Reference genes' in Appendix I: cDNA synthesis).
If the amplification efficiency of a reference gene is the same as that of the target gene, the comparative $\Delta\Delta$Cq method can be used for relative quantification. Both the sample and the calibrator data are first normalized against variation in sample quality and quantity. Normalized (ΔCq) values are calculated by the following equations:
Normalized (ΔCq) values are calculated by the following equations:

ΔCq(sample) = Cq(target) – Cq(reference)
ΔCq(calibrator) = Cq(target) – Cq(reference)

The $\Delta\Delta$Cq value is then determined using the following formula:

$\Delta\Delta$Cq = ΔCq(sample) –ΔCq(calibrator)

The expression of the target gene normalized to the reference gene and relative to the calibrator = $2^{-\Delta\Delta Cq}$

If the amplification efficiency of a reference gene is not the same as that of the target gene, a method should be used that takes this into account (Pfaffl MW. (2001) *Nucleic Acids Res.* 29: e45).

TROUBLESHOOTING

	Possibles causes	Comments and suggestions
No increase in fluorescence signal	Error in cycler setup	• Make sure that the instrument settings are correct for the experiment.
	Missing components (e.g. primers or template) or pipetting error	• Check the assembly of the reactions. • Check the concentrations and storage conditions of the reagents.
	Missing essential step in the cycler protocols	• Check the cycler protocol.
	qPCR primer and/or probe design or concentration not optimal	• Re-check primer and probe design. See Section 4.4. • Use primer concentration of 0.5 µM and probe concentration of 0.25 µM if not otherwise optimised.
	Improperly stored or expired reagents	• Check storage conditions and expire dates of the reagents.
	Sample not configured properly	• Check the plate configuration.
Late increase in fluorescence signal	Error in cycler setup	• Make sure that the instrument settings are correct for the experiment.
	Insufficient starting template	• Check the calculation of the template stock concentration; increase the template amount if possible.
	Improperly stored or expired reagents	• Check storage conditions and expiration dates of the reagents.
	qPCR primer and/or probe design not optimal	• Check primer and probe design.
	Insufficient extension time for the amplicon size	• Increase extension time.
	Primer and probe concentration too low	• Increase primer concentration (to a maximum of 1 µM each). 0.25 µM probe concentration is usually sufficient.
	Insufficient activation of the hot start *Tbr* DNA polymerase	• Make sure 95°C 7 min was used for the initial denaturation step. • Make sure cycler block temperature is accurate.
	PCR protocol not optimal	• Make sure you are using the recommended PCR protocol. If necessary, optimize using the recommended protocol as a starting point.

	Possibles causes	Comments and suggestions
Normal fluorescence signal, but low efficiency	Pipetting error	• Check the assembly of the reactions.
	Primer-dimers from a previous run contaminating the reaction	• Perform UDG treatment before PCR cycling.
	Primer and probe design not optimal	• Check primer and probe design.
	Inhibitors from the sample affecting reaction	• Repurify DNA.
	Low initial template concentration	• Increase template amount.
Non-linear correlation between Cq and log of template amount in the standard curve	Template dilution inaccurate	• Remake dilution series and make sure the samples are well mixed.
	Template amount too high	• Reduce the template amount. • Increase reaction volume.
	Template amount too low	• Increase the template amount.
	Insufficient activation of the hot start *Tbr* DNA polymerase	• Make sure 95°C 7 min was used for the initial denaturation step. • Make sure cycler block temperature is accurate.
	Insufficient denaturation of template	• Make sure 95°C 7 min was used for the initial denaturation step. • Make sure cycler block temperature is accurate.
	Serious contamination	• Find contamination source and change contaminated components.
When using the blue master mix and yellow sample dye, reactions remain blue after sample addition instead of turning green	Insufficient concentration of the sample dye	• Use 1X concentration in the final reaction. For example 4X in a 5 µL sample when the total reaction volume will be 20 µL. See Section 4.8.
Low signal when using ROX normalization	High ROX passive reference fluorescence intensity	• Use lower ROX concentration. See recommended concentrations in Table 2.
High signal when using ROX normalization	Low ROX passive reference fluorescence intensity	• Use higher ROX concentration. See recommended concentrations in Table 2.
	Yellow dye in the sample buffer decreases ROX intensity	• Use higher ROX concentration. See recommended concentrations in Table 2.
Abnormal appearance of amplification curves when ROX normalization is used	Color calibration not accurate. Fluorescence intensity from one channel affects intensity in another channel.	• Verify color calibration according to instrument instructions.

Appendix I: cDNA synthesis

The cDNA synthesis step is very critical in RT-qPCR. The efficiency of reverse transcription varies and can be low in some cases. The expression level of the target RNA molecule and the efficiency of the RT reaction must therefore be considered when determining the appropriate amount of the starting template for subsequent PCR steps. The volume of cDNA template should not exceed 10% of the qPCR reaction volume, as elevated volumes of the template may reduce the efficiency of the PCR amplification. A dilution series of the template can be done to optimize the volume of the starting material used.
Since RNA quantification involves a number of variables, and each experiment is inherently different, careful experiment design is very important. Useful information and guidelines for experiment design, normalization, RNA standards, etc. can be found in the following review articles:

Bustin S.A. (2000) *Journal of Molecular Endocrinology* 25: 169–193
Bustin S.A. (2002) *Journal of Molecular Endocrinology* 29: 23–39.

We recommend using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (K1641) or DyNAmo cDNA Synthesis Kit (F-470) for the reverse transcription step. These kits have been specifically optimized for quantitative reverse transcription.

RT Primers

Random hexamers, oligo(dT) or specific primers can be used for the RT step. A good starting point is to use random hexamers for cDNA synthesis. Random hexamers transcribe all RNA, producing cDNA that covers the whole transcript. Oligo(dT) primers can be used to transcribe poly(A)+ RNAs, and gene-specific primers to transcribe only the particular RNA of interest. Using specific primers can help to decrease background. Random hexamers and oligo(dT) primers are useful if several different amplicons need to be analyzed from a small amount of starting material.

Primers for qPCR step

PCR primers in qRT-PCR experiments should be designed to anneal to sequences in two exons on opposite sides of an intron. A long intron inhibits the amplification of the genomic target. Alternatively, primers can be designed to anneal to the exon-exon boundary of the mRNA. With such an assay design, the priming of genomic target is highly inefficient.

DNase I

If primers cannot be designed to anneal to the exon-exon boundaries or in separate exons, the RNA sample must be treated with RNase-free DNase I.

Minus RT control

A minus RT control should be included in all qRT-PCR experiments to test for DNA contamination (such as genomic DNA or PCR product from a previous run). Such a control reaction contains all the reaction components except for the reverse transcriptase. RT reaction should not occur in this control, so if PCR amplification is seen, it is most likely derived from contaminating DNA.

Reference genes

When studying gene expression, the quantity of the target gene transcript needs to be normalized against variation in the sample quality and quantity between samples. To ensure identical starting conditions, the relative expression data have to be normalized with respect to at least one variable, such as sample size, total amount of RNA, or reference gene(s), for example. A gene used as a reference should have a constant expression level that is independent of the variation in the state of the sample tissue. Examples of commonly used reference genes are beta actin, GAPDH and 18S rRNA. A problem is that, even with housekeeping genes, the expression usually varies to some extent. That is why several reference genes are usually required, and their expression needs to be checked for each experiment. For relative quantification ($\Delta\Delta$Cq method), see Section 6.2.
The amplification efficiency of a reference gene should be the same as the amplification efficiency of the target gene, i.e. the slopes of their standard curves are the same. For efficiency calculation using the slope, see Section 6.1 (Absolute quantification).

Appendix II: general molecular biology data

Table 7. Spectrophotometric conversions for nucleic acid templates.

1 A_{260} unit*	Concentration (µg/mL)
Double-stranded DNA	50
Single-stranded DNA	33
Single-stranded RNA	40

* Absorbance at 260 nm = 1 (1 cm detection path).

Table 8. Molar conversions for nucleic acid templates.

Nucleic acid	Size	pmol/µg	Copies/µg*
1 kb DNA	1000 bp	1.52	9.1×10^{11}
pUC19DNA	2686 bp	0.57	3.4×10^{11}
Lambda DNA	48502 bp	0.03	1.8×10^{10}
Escherichia coli	4.7×10^{6} bp	3.2×10^{-4}	1.9×10^{8}
Human	3.2×10^{9} bp	4.7×10^{-7}	2.8×10^{5}

* For single-copy genes.

NOTICE TO PURCHASE:

- The purchase price of this product includes a limited, non-transferable license under U.S. and foreign patents owned by BIO-RAD Laboratories, Inc., to use this product. No other license under these patents is conveyed expressly or by implication to the purchaser by the purchase of this product.
- Use of this product is covered by US Patent No. 6,127,155. Use of this product in a passive reference method is covered by the following US Patent No. 5,928,907 (claim numbers 12-24, 27-28) and corresponding patent claims outside the US. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim, no right to perform any patented method and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

Patent Note

This product or the use of this product is covered by US patent application US20110251080A1 and corresponding counterparts. The purchase of this product includes a non-transferable license to use this product for the purchaser's internal research. All other commercial uses of this product, including without limitation product use for diagnostic purposes, resale of product in the original or any modified form or product use in providing commercial services require a separate license. For further information on obtaining licenses please contact ltvil.info@thermofisher.com

PRODUCT USE LIMITATION

This product is developed, designed and sold exclusively for research purposes and *in vitro* use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.

Please refer to www.thermoscientific.com/onebio for Material Safety Data Sheet of the product.