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PRODUCT INFORMATION

Thermo Scientific

DyNAmo Flash SYBR Green qPCR Kit

DyNAmo ColorFlash SYBR Green qPCR Kit

#F-416S

Lot 00000000 Expiry Date ____

Store at -20°C

37

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Contents

COMPONENTS OF THE KIT

DyNAmo Flash SYBR Green qPCR Kit	**#F-415S**	**#F-415L**	**#F-415XL**
2X master mix (contains hot-start version of a modified *Tbr* DNA polymerase, SYBR® Green I, optimized PCR buffer, 5 mM $MgCl_2$, 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 SYBR Green qPCR Kit	**#F-416S**	**#F-416L**	**#F-416XL**
2X master mix with blue dye (contains hot-start version of a modified *Tbr* DNA polymerase, SYBR® Green I, optimized PCR buffer, 5 mM $MgCl_2$, 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 x 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 SYBR Green qPCR Kit and the Thermo Scientific™ DyNAmo™ ColorFlash SYBR Green 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 SYBR Green 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 SYBR Green qPCR Kit (F-415) and DyNAmo ColorFlash SYBR Green qPCR Kit (F-416) are designed for quantitative, real-time analysis of DNA samples from various sources. 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 SYBR Green qPCR Kits is based on a hot-start version of a modified *Thermus brockianus* DNA polymerase and SYBR Green I fluorescent dye. A nonspecific DNA binding domain has been fused to the *Tbr* DNA polymerase. This domain lends physical stability to the polymerase-DNA complex. The initial denaturation step in the PCR protocol activates the modified hot-start *Tbr* polymerase. SYBR Green I is specific for double-stranded DNA and fluoresces when bound to the amplified double-stranded PCR product, thereby permitting the direct quantification of amplified DNA without labeled probes. The buffer composition of DyNAmo Flash SYBR green qPCR master mix is specificly optimized for shorter annealing and extension times without compromising the qPCR performance.

The qPCR master mix in DyNAmo ColorFlash SYBR Green qPCR Kit (F-416) contains a blue dye that helps keeping track of pipetting of the master mix into the reaction wells. Additionally, a sample buffer with a yellow dye is provided to ease the pipetting of the samples. Adding the yellow sample dye is optional, but by using the dye it is very easy to see in which wells the 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 SYBR Green qPCR Kits is applicable to most real-time PCR instruments, including those from Applied Biosystems, Bio-Rad Laboratories, Corbett Research, and Stratagene. When RNA is used as the starting material for producing cDNA, we recommend Thermo Scientific™ Maxima™ First Strand cDNA Synhesis Kit for RT-qPCR (K1641) or Thermo Scientific™ DyNAmo™ cDNA synthesis Kit (F-470) to ensure high-quality results.

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 100 ng of genomic DNA in a 20 µL reaction.
Primer 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).
$MgCl_2$	1X master mix contains 2.5 mM $MgCl_2$

4.1. qPCR master mix

The 2X qPCR master mix in the DyNAmo Flash SYBR Green qPCR Kits includes a hot-start version of a modified *Thermus brockianus* DNA polymerase. The modified polymerase incorporates a nonspecific DNA binding domain that lends physical stability to the polymerase-DNA complex. The modified *Tbr* 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. The reaction setup can be performed at room temperature. The initial denaturation step in the PCR protocol reactivates the polymerase (hot start).

The 2X qPCR master mix in DyNAmo Flash SYBR Green qPCR Kit (F-415) is colorless, but the master mix in DyNAmo ColorFlash SYBR Green qPCR Kit (F-416) 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. PCR primers

Careful primer design is particularly important to minimize nonspecific primer annealing and primer-dimer formation, since fluorescence from SYBR Green I increases strongly upon binding to any double-stranded DNA. Standard precautions must be taken during primer design to avoid primer-dimer or hairpin loop formation. Most primer design software tools will yield well-designed primers for use in qPCR. In most cases, good results are obtained using a concentration of 0.5 µM for each primer. The optimum primer concentration is usually between 0.3 and 1 µM.

4.3. 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.

4.4. Standards

Standard curve is needed for absolute quantification and for analyzing the efficiency of the qPCR reaction (see Section 6.2). 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 binding sites are identical to ensure equivalent amplification efficiencies of both standard and target molecules.

4.5. 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 SYBR Green qPCR Kit (F-416) the fluorescence intensity of ROX can be lower than with DyNAmo Flash SYBR Green qPCR Kit (F-415), 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)

4.6. 40X Sample Buffer with yellow dye (F-416 only)

The 40X Sample Buffer with yellow dye is included in DyNAmo ColorFlash SYBR Green qPCR Kit (F-416). It is used to track pipetting of samples to the qPCR reactions. When using the blue master mix of the DyNAmo ColorFlash SYBR Green 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.

4.7. 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 containing reaction products should not be opened or analyzed by gel electrophoresis in the same laboratory area that is used to set up reactions.

4.8. Reaction volume

A reaction volume of 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.

4.9. 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 Synhesis 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 DNA polymerase is inactive during PCR setup, it is not necessary to do the setup on ice.
- Pipette with sterile filter tips.
- Minimize the exposure of the qPCR master mix to light.
- 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 SYBR Green qPCR Kit (F-416) 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 and master mix (and the ROX passive reference dye, if necessary, see Section 5.2). 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, (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 (< 100 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.

Components (In order of addition)	**20 µL reaction**	**50 µL reaction**	**Final concentration**	**Comments**
2x Master mix	10 µL	25 µL	1X	Mix thoroughly.
Primer mix (in H_2O)	X µL	X µL	0.5 µM fwd 0.5 µM rev	Titrate from 0.3 to 1 µM if necessary.
50X ROX reference dye	(0.12–0.4 µL)	(0.3–1 µL)	0.3–1X	Optional (see Section 4.5 and 5.2).
Template DNA (including yellow sample buffer)	X µL	X µL		Do not exceed 5 ng/µl in the final reaction. Using the yellow sample buffer is optional.
H_2O	add to 20 µL	add to 50 µL		

For different volumes, adjust all components proportionally.

Cycling protocol

Table 4. Cycling protocol.

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	10 s	
	Annealing			Optional. If separate annealing is required, see page 12.
3*	Annealing/ extension	60°C (72°C)	15-30 s	With fast ramping instruments, use longer incubation time. If a separate annealing step is performed, temperature up to 72°C can be used for extension.
4	Data acquisition			Fluorescence data collection
5	Number of cycles	35-45 cycles, steps 2-4		
	Final extension			Optional, see page 13.
6	Melting curve	60-98°C	As instructed by the instrument manufacturer	Note that melting curve setting options vary between different real-time instruments. See the instrument manufacturer's manual for detailed information.

* 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).
If genomic DNA is used as a template, use a 30 s annealing/extension time.

UDG incubation (optional)

If UDG enzyme is used, incubate as instructed by the UDG manufacturer. This step does not negatively affect qPCR performance because the hot-start DNA polymerase is not active at UDG incubation temperature.

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 10 s is sufficient in most cases.

Annealing/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. Due to the unique characteristics of the modified hot start DNA polymerase it is often possible to use higher annealing temperatures than with other enzymes and thereby minimize the chances of primer-dimer formation or amplification of nonspecific products. 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 seconds to allow complete amplification in every cycle. When genomic DNA is used as a template, an extension time of 30 seconds is recommended.

If the primers cannot be designed to anneal efficiently at 60°C, the annealing and extension steps can be performed separately. The annealing temperature should be 5°C below Tm, and annealing time 15 seconds. For most reactions the extension temperature should be between 60°C and 72°C. Make sure that the extension temperature is lower than the melting point of the PCR product.

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 the nearest-neighbor 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.

Data acquisition

Data acquisition is normally performed at extension temperature. If significant amounts of primer-dimers are co-amplified with the specific product, it may be helpful to perform a data acquisition step at an elevated temperature to minimize the interference of primer-dimers with quantification (Morrison, T.B. *et al. (1998) Biotechniques* 24: 954–62). The temperature used should be sufficiently higher than the Tm of any primer-dimer (usually < 80°C) and lower than that of the specific product.

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. An excessive number of cycles can lead to nonspecific amplification, which manifests itself in undesirable products seen during melting curve analysis.

Final extension (optional)

A final extension is performed to ensure that all amplification products are in double-stranded form before the melting curve step. The temperature in the final extension step should be equal to the starting temperature of melting curve analysis.

Melting curve

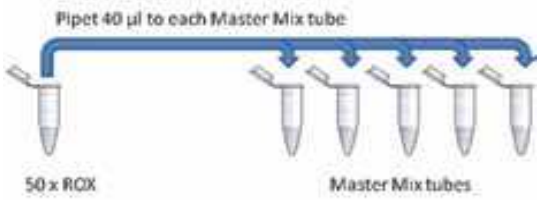
A melting curve is used to check the specificity of an amplified product. When the temperature is gradually increased, a sharp decrease in SYBR Green fluorescence is observed as the product undergoes denaturation. Specific products can be distinguished from the nonspecific products by the difference in their melting temperatures. The recommended temperature ramp time is stated in Table 4. If a faster protocol is preferred, the ramp time of the melting curve can be increased with some instruments, although this may affect resolution. If there is a need to check for possible low-melting products, the starting temperature of the melting curve can be lowered from 72°C to 65°C, for example. In that case, the final extension should also be performed at the same temperature.

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 SYBR Green qPCR Kit (F-416) and wish to track pipetting when adding samples to the reactions.

- Program the cycler as outlined in Table 6.
- Thaw the template DNA, primers and 2X master mix (to which ROX passive reference dye has been added). Mix the individual solutions to ensure homogeneity. This is especially important for the master mix.
- 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.
- Prepare a PCR premix by mixing 2X master mix, primers, and H_2O. Mix the PCR premix thoroughly to ensure homogeneity. Dispense appropriate volumes into strip tubes or plate wells. Use the reverse pipetting technique to avoid bubbles.
- 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) should not exceed 10% of the final PCR volume.
- Seal the strips or plate with an 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	
Template DNA (including yellow sample buffer)	X µL	X µL		Do not exceed 5 ng/µL in the final 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	10 s	40 cycles
Annealing/extension	60°C	30 s*	
Dissociation curve as instructed by the instrument manufacturer			

* A shorter annealing/extension step (down to 15 s) can be used with the following instruments: ABI 7000, 7700 and 7900. For additional information, refer to Section 5.1.

6. ANALYSIS

6.1. Melting curve

Melting curve analysis is typically included in the analysis software of real-time fluorescence detection instruments. The melting point of the product depends mainly on base composition and length. When the decrease in SYBR Green fluorescence during the temperature increase is plotted as a negative first derivative, the temperature of the peak is defined as the Tm, or the melting temperature of the product.
If primer-dimers or other nonspecific products are observed, the efficiency of the PCR should be checked. Varying efficiency leads to incorrect quantification.

6.2. 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.

6.3. 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 ΔΔCq 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 ΔΔCq value is then determined using the following formula:

ΔΔCq = 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 design or concentration not optimal	• Re-check primer design. See Section 4.2. • Use primer concentration of 0.3–1.0 µM.
	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.
	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.
	Insufficient activation of the hot start DNA polymerase	• Make sure 95°C 15 min was used for the initial reactivation/ denaturation step. • Make sure the cycler block temperature is accurate.
	Template amount too low	• Check the calculation of the template stock concentration; increase the template amount if possible.
	qPCR primer design not optimal	• Re-check primer design. See Section 4.2.
	qPCR primer concentration too low	• Increase primer concentration (to a maximum of 1 µM each).
	Annealing temperature too high	• Use a gradient to optimize the annealing temperature. • Decrease the annealing temperature in 2°C decrements if no gradient feature is available.
	Insufficient extension time for the amplicon size	• We recommend 30 s extension time for < 500 bp amplicons.
	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 melting curve analysis shows primer-dimers or nonspecific products only	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.
	Primer-dimers from a previous run contaminating the reaction	• Perform UDG treatment before PCR cycling.
	Annealing temperature too low	• Use gradient to optimize annealing temperature. • Increase annealing temperature in 2°C increments if no gradient feature is available.
	qPCR primer design not optimal	• Re-check primer design. See Section 4.2.
Normal fluorescence signal, melting curve analysis shows both primer-dimer or nonspecific product and specific product peaks	Template amount too low	• Increase template amount.
	qPCR primer design not optimal	• Re-check primer design. See Section 4.2.
	Primer concentration too high	• Optimize primer concentration. Titrate from 0.3 to 1 µM.
	Annealing temperature too low	• Use gradient to optimize annealing temperature. • Increase annealing temperature in 2°C increments if a gradient feature is not available.
	Primer-dimers or PCR products from previous run contaminating the reaction	• Perform UDG treatment before PCR cycling.
	Co-amplification of primer-dimers with the specific product	• Perform a second data acquisition at an elevated temperature to minimize the interference of primer-dimers.
	Extension time too long	• Decrease extension time.
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.
	Template amount too low	• Increase the template amount.
	Insufficient activation of the hot start DNA polymerase	• Make sure 95°C 15 min was used for the initial reactivation/denaturation step in qPCR. • Make sure the cycler block temperature is accurate.
	Co-amplification of primer-dimers with the specific product	• Perform a second data acquisition at an elevated temperature to minimize the interference of primer-dimers.
	qPCR primer design or concentration not optimal	• Re-check primer design. See Section 4.2. • Use primer concentration of 0.3–1.0 µM.

	Possibles causes	Comments and suggestions
High initial fluorescence signal, gradually decreasing over the first 10–20 cycles	Template amount too high	• Reduce the template amount.
	Insufficient denaturation of template	• Make sure 95°C 10 min is used for the initial denaturation step in qPCR. • Make sure the cycler block temperature is accurate.
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 4.6.
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 qRT-PCR. 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 6.3.
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 6.2 (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.

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