

# DyNAmo™ Flash SYBR® Green qPCR Kit

## Instruction manual

<b>F-415S</b>	40 reactions (50 µl each) or 100 reactions (20 µl each)
<b>F-415L</b>	200 reactions (50 µl each) or 500 reactions (20 µl each)
<b>F-415XL</b>	1000 reactions (50 µl each) or 2500 reactions (20 µl each)

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# 1. Description

Finnzymes' DyNAmo™ Flash SYBR® Green qPCR Kit is designed for fast real-time quantitative PCR. Quantitative PCR (qPCR) is a useful technique for the investigation of gene expression, viral load, pathogen detection, and numerous other applications. Fast protocols increase instrument throughput, because more runs can be done in the same time. Fast assays are especially important when sample processing, qPCR run and data analysis should be done during the same day.

The performance of the DyNAmo Flash SYBR Green qPCR Kit is based on a hot start version of a modified *Thermus brockianus* DNA polymerase and SYBR Green I fluorescent dye. A DNA-binding domain attached to the polymerase in this kit improves the physical stability of the polymerase-DNA complex. The initial denaturation step in the PCR protocol activates the modified hot start *Tbr* polymerase. SYBR Green I is a dye specific for double-stranded DNA and fluoresces when bound to the amplified double-stranded PCR product, thereby enabling direct quantitation of amplified DNA without labeled probes. The buffer composition of DyNAmo Flash SYBR green qPCR master mix is specially optimized for shorter annealing and extension times without compromising the qPCR performance.

The reaction chemistry of DyNAmo Flash SYBR Green qPCR Kit is suitable for most real-time PCR instruments, e.g. from Applied Biosystems, Bio-Rad Laboratories, Corbett Research, and Stratagene. For capillary-based instruments, e.g. LightCycler™ (Roche), we recommend DyNAmo™ Capillary SYBR® Green qPCR Kit (F-420). When using RNA as starting material, we recommend DyNAmo cDNA Synthesis Kit (F-470) for producing cDNA in order to ensure high-quality results.

# 2. Kit components

DyNAmo Flash SYBR Green qPCR Kit	F-415S	F-415L	F-415XL
2x master mix (contains hot start version of a modified <i>Tbr</i> DNA polymerase, SYBR Green I, optimized PCR buffer, 5 mM MgCl <sub>2</sub> , dNTP mix including dUTP)	1 x 1 ml (sufficient for 40 reactions of 50 µl or 100 reactions of 20 µl)	5 x 1 ml (sufficient for 200 reactions of 50 µl or 500 reactions of 20µl)	25 x 1 ml (sufficient for 1000 reactions of 50 µl or 2500 reactions of 20µl)
50x ROX passive reference dye	1 x 50 µl	1 x 250 µl	1 x 1.25 ml

### 3. Shipping and storage

The DyNAmo Flash SYBR Green qPCR Kit is shipped on gel ice. Upon arrival, store all kit components at -20 °C. 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 kit is stable for six months from the date of packaging when stored and handled properly.

### 4. General considerations

**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 genomic DNA in a 20 µl reaction.
Primer design	Use primers with matched T <sub>m</sub> . Avoid inter- and intra-primer complementary sequences. (T <sub>m</sub> is recommended to be calculated with the nearest-neighbor method as described by Breslauer <i>et al.</i> , (1986) <i>Proc. Nat. Acad. Sci.</i> 83, 3746–50.) Instructions for T <sub>m</sub> calculation and a link to a calculator using the nearest-neighbor method can be found on Finnzymes' website ( <a href="http://www.finnzymes.com">www.finnzymes.com</a> ).
MgCl <sub>2</sub>	1x master mix contains 2.5 mM MgCl <sub>2</sub> .

#### 4.1 DNA polymerase

The 2x qPCR master mix in the DyNAmo Flash SYBR Green qPCR Kit includes a hot start version of a modified *Thermus brockianus* DNA polymerase. A DNA-binding domain attached to the polymerase improves the physical stability of 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).

## 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 the binding of the dye to any double-stranded DNA. Standard precautions must be used 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. Typically, good results are obtained using a concentration of 0.5  $\mu\text{M}$  for each primer. The optimum primer concentration is usually between 0.3 and 1  $\mu\text{M}$ .

## 4.3 Template preparation and quality

Purity of nucleic acid templates is particularly important for qPCR, because 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 quantitation and for analyzing the efficiency of the qPCR reaction (see Absolute quantitation on page 12). 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 C(t) values to external standards containing a known quantity of DNA. (C(t) = cycle threshold, the cycle number at which the fluorescence signal reaches the threshold level above background. The threshold level 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 the ROX passive reference dye needed can vary depending on the type of excitation. More ROX dye may be needed with real-time cyclers that use argon laser as excitation light source or have excitation filters not optimal for ROX dye than instruments that excite efficiently near 585 nm.

The ROX dye is provided as a 50x solution dissolved in a buffer that is compatible with the qPCR reaction buffer. The optimal ROX dye concentration is usually at 0.3–1x concentration (see table 2 for instrument specific recommendations).

**Table 2. ROX concentration**

Real-time PCR instrument	Recommended ROX concentration
ABI PRISM® 7000 Sequence Detection System	1x
ABI PRISM® 7700 Sequence Detection System	1x
ABI PRISM® 7900HT Sequence Detection System	1x
Applied Biosystems 7300 Real-Time PCR System	1x
Applied Biosystems 7500 Real-Time PCR System	0.3x
Stratagene Mx3000P® QPCR System	0.3x
Stratagene Mx3005P® QPCR System	0.3x
Stratagene Mx4000® QPCR System	0.3x

## 4.6 UNG (UDG) 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 UNG prior to PCR. UNG (uracil-N-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). UNG is inactivated during the first denaturation step in PCR. The UNG treatment step has no negative effect on qPCR performance because the hot start *Tbr* DNA polymerase is not reactivated at UNG incubation temperatures. All Finnzymes' DyNAmo qPCR Kits contain dUTP and therefore UNG treatment can be used.

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 for reaction setup.

## 4.7 Reaction volume

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

## 4.8 Quantitation of RNA

To determine the quantity of RNA, a reverse transcription (RT) reaction must be performed prior to qPCR. Finnzymes offers DyNAmo™ cDNA Synthesis Kit (F-470) for quantitative reverse transcription. DyNAmo Flash SYBR Green qPCR kit has been optimized using the DyNAmo cDNA Synthesis kit.

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 calculating the appropriate amount of starting template for subsequent PCR steps. The volume of cDNA template should not exceed 10 % of the qPCR reaction volume, as elevated volumes of cDNA reaction components may reduce the efficiency of the PCR amplification. A dilution series of the template can be prepared to optimize the volume of the starting material.

Since RNA quantification involves a number of variables, and each experiment is inherently different, careful experimental design is very important. Useful information and guidelines for experimental design, normalization, RNA standards, etc. can be found in the following review articles:

Bustin, S.A. *Journal of Molecular Endocrinology* 25, 169–193 (2000).

Bustin, S.A. *Journal of Molecular Endocrinology* 29, 23-39 (2002).

### 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)<sup>+</sup> RNAs, and gene-specific primers to transcribe only the particular RNA of interest. Using specific primers can help decreasing 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 experiment should be designed to anneal to sequences in two exons on opposite sides of an intron. Long intron inhibits amplification of genomic target. Alternatively, primers can be designed to anneal to the exon-exon boundary of the mRNA. With such assay design priming of genomic target is highly inefficient.

### DNase I

DNase I treatment of the RNA sample removes contaminating genomic DNA and it should be performed especially if primers cannot be designed in the exon-exon boundaries or in separate exons.

## Minus RT control

A minus RT control should be included in all qRT-PCR experiments to test for DNA contamination (for example genomic DNA or PCR product from a previous run). It 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 the quantity of a reference gene transcript in the same sample. Examples of commonly used reference genes are beta-actin, GAPDH and 18S rRNA. A gene used as a reference should have a constant expression level independent of the variation in the state of the sample tissue. A problem is that even housekeeping genes are to some extent variable in their expression. That is why several reference genes are usually required, and their expression needs to be assayed in each experiment. For  $\Delta\Delta C(t)$  method for relative quantitation, see page 12.

The amplification efficiency of a reference gene should be the same as the efficiency of the target gene, i.e. the slopes of their standard curves are the same. For efficiency calculation using the slope, see chapter "Absolute quantitation" on page 12. If the amplification efficiency of a reference gene is not the same as the efficiency of the target gene, the results have to be corrected for the efficiency (Pfaffl MW., (2001) *Nucleic Acids Res.* 29: e45).

# 5. Reaction setup

- Perform the reaction setup in an area separate from nucleic acid preparation or PCR product analysis.
- As the hot start DNA polymerase is inactive at room temperature, it is not necessary to do the setup on ice.
- Make sure that all the reaction components are properly mixed
- Pipet with sterile filter tips.
- Minimize pipetting errors by using calibrated pipettes and by preparing premixes in order to avoid pipetting very small volumes.
- Use optically clear caps or sealers to achieve maximal signal.
- Use a cap sealing tool, a film sealer or firm finger pressure to properly close caps.
- 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.
- Use molecular biology grade H<sub>2</sub>O.

## 5.1 Protocol

1. Program the cycler as outlined in table 4.
2. Thaw template DNA, primers and master mix (and ROX passive reference dye if necessary). Mix the individual solutions to assure homogeneity. This is especially important for the master mix.
3. Prepare a PCR premix by mixing master mix, primers, (ROX) and H<sub>2</sub>O. Mix the PCR premix thoroughly to assure homogeneity. Dispense appropriate volumes into strip tubes or plate wells.
4. Add template DNA (<100 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.
5. Place the strips or plate in the thermal cycler and start the cycling program.

**Table 3. Reaction setup.**

Components (In order of addition)	50 µl reaction	20 µl reaction	Final concentration	Comments
2x Master mix	25 µl	10 µl	1x	Mix thoroughly.
Primer mix (in H <sub>2</sub> O)	X µl	X µl	0.5 µM fwd 0.5 µM rev	
50x ROX reference dye	(0.03–1 µl)	(0.012–0.4 µl)	0.03–1x	Optional (see page 4).
Template DNA (in H <sub>2</sub> O)	X µl	X µl		Do not exceed 10 ng/µl in the final reaction
H <sub>2</sub> O	add to 50 µl	add to 20 µl		

For different volumes, adjust all components proportionally.

## 6. Cycling protocol

**Table 4. Cycler protocol.**

Step	Purpose	Temp	Time	Comments
1 <sup>1</sup> (optional)	UNG incubation	X°C	X	Incubate as instructed by the UNG manufacturer.
2	Initial denaturation	95°C	7 min	This step is needed to activate the hot start DNA polymerase and to denature the template DNA.
3	Denaturation	95°C	10 s	
4 (optional)	Annealing	X°C	15 s	If primers cannot be designed to anneal efficiently at 60°C, use separate annealing at 5°C below T <sub>m</sub> .
5 <sup>2, 3</sup>	Annealing/extension	60°C (72°C)	15–30 s	With fast ramping instrument, use longer incubation time. If separate annealing step is performed, use 72°C for extension.
6	Data acquisition			Fluorescence data collection
7 <sup>4</sup> (optional)	Data acquisition	X°C	1 s	T <sub>m</sub> (primer-dimer) < X < T <sub>m</sub> (product); Use melting curve analysis to find the appropriate temperature. Fluorescence data collection at higher than extension temperature prevents errors in case primer-dimers are observed.
8	Number of cycles	35-45 cycles, steps 3-6		
9	Final extension (optional)	60°C	1 min	Final extension ensures that all amplification products are in a double-stranded form before melting curve step.
10	Melting curve	60-98°C	As instructed by the instrument manufacturer.	Note that melting curve setting options vary between different real-time instruments. See instrument manufacturer's manual for detailed information.

- Step 1 can be excluded if UNG is not used.
- Use the T<sub>m</sub> calculator at [www.finnzymes.com](http://www.finnzymes.com) to determine T<sub>m</sub> of the primers. Use 50 mM KCl and 500 nM primer concentration when calculating T<sub>m</sub> (or the primer concentration in your reaction if optimized to other than 500 nM). Design primers so that they are efficiently annealed at 60°C (T<sub>m</sub> should be about 65°C).
- If genomic DNA is used as a template, use 30 s annealing/extension time.
- Step 7 is recommended if significant amounts of primer-dimers are co-amplified with the specific product (Morrison, T.B. *et al.*, (1998) *Biotechniques* 24: 954-62).

## 6.1 Cycling steps

### UNG incubation

If UNG enzyme is used, incubate as instructed by the UNG manufacturer (step 1). This step does not negatively affect qPCR performance, because the hot start DNA polymerase is not activated at UNG incubation temperature.

### Initial denaturation/reactivation

Initial denaturation (step 2) at 95°C for 7 min is needed to assure efficient reactivation of the hot start DNA polymerase and complete denaturation of the template.

### Denaturation

Denaturation (step 3) at 95°C for 10 s is sufficient in most cases.

### Annealing/extension

For most amplicons, combined annealing and extension (step 5) for 15 s at 60°C works well if 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 s to allow complete amplification in every cycle. When genomic DNA is used as a template, an extension time of 30 s 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  $T_m$ . The extension temperature should be between 60 and 72°C for most reactions. In cases where the melting point of the product is near or lower than 72°C, a lower extension temperature (e.g. 68°C) should be used.

These guidelines are based on  $T_m$  values calculated (50 mM salt and 500 nM primer) with the nearest-neighbor method. Instructions for  $T_m$  calculation and a link to a calculator using the nearest-neighbor method can be found on Finnzymes' website ([www.finnzymes.com](http://www.finnzymes.com)). A temperature gradient may be used to find the optimal annealing temperature for each starting template-primer pair combination.

### **Data acquisition**

If primer-dimers are observed in the melting curve, it may be helpful to perform a data acquisition step at an elevated temperature (step 7) to minimize the interference of primer-dimers with quantitation. The temperature used should be sufficiently above the  $T_m$  of any primer-dimer (usually  $< 80\text{ }^\circ\text{C}$ ) and below that of the specific product.

### **Number of cycles**

35-45 cycles of amplification (step 8) is sufficient for most applications even when the template is present at a very low copy number. An exceedingly high number of cycles can lead to nonspecific amplification, as evidenced by undesirable products seen during melting curve analysis.

### **Final extension**

A final extension (step 9) is performed to ensure that all amplification products are in a 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 (step 9).

### **Melting curve**

A melting curve (step 10) is performed to check the specificity of an amplified product. Melting curve step should be done as instructed by the instrument manufacturer. If a faster protocol is preferred, the ramp time of the melting curve can be increased with some instruments, however this may affect resolution..

## **7. Analysis**

### **7.1 Melting curve**

Melting curve analysis is typically included in the analysis software of real-time fluorescence detection instruments. With a melting curve analysis, specific products can be distinguished from nonspecific products by the difference in their melting temperatures. When the temperature is gradually increased, a sharp decrease in SYBR Green fluorescence is observed as the product undergoes denaturation. The melting point of the product depends mainly on base composition and length.

If primer-dimers or other nonspecific products are observed in the melting curve, the efficiency of the PCR should be checked using a standard curve (see chapter "Absolute quantitation"). Varying efficiency leads to incorrect quantitation.

## 7.2 Absolute quantitation

Absolute quantitation is performed by plotting samples of unknown concentration to a standard curve generated from a dilution series of template DNA of known concentration. Typically, the standard curve is a plot of the threshold cycle (C(t)) 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 quantitation to be accurate. The PCR efficiency of the samples can be determined by doing a dilution series of these samples.

For a graph where C(t) is on the y-axis and log(DNA copy#) on the x-axis:

$$\text{PCR efficiency} = ((10^{\frac{-1}{\text{slope}}}) - 1) \times 100\%$$

The slope of  $-3.322$  equals 100% efficiency.

For a graph where log(DNA copy#) is on the y-axis and C(t) on the x-axis:

$$\text{PCR efficiency} = ((10^{-1 \times \text{slope}}) - 1) \times 100\%$$

The slope of  $-0.301$  equals 100% efficiency.

## 7.3 Relative quantitation

Relative quantitation is used to determine the ratio between the quantity of a target molecule in a sample and in the calibrator (calibrator being e.g. healthy tissue or untreated cells). The most common application of this method is the analysis of gene expression, e.g. comparisons of gene expression levels in different samples. Target molecule quantity is usually normalized with a reference gene. See chapter "reference genes" on page 7.

If the amplification efficiency of a reference gene is the same as the efficiency of the target gene, the comparative  $\Delta\Delta C(t)$  method can be used for relative quantitation. Both the sample and the calibrator data is first normalized against variation in sample quality and quantity. Normalized values,  $\Delta C(t)$ s, are first calculated from following equations:

$$\Delta C(t)_{\text{sample}} = C(t)_{\text{target}} - C(t)_{\text{reference}}$$

$$\Delta C(t)_{\text{calibrator}} = C(t)_{\text{target}} - C(t)_{\text{reference}}$$

The  $\Delta\Delta C(t)$  is then determined using the following formula:

$$\Delta\Delta C(t) = \Delta C(t)_{\text{sample}} - \Delta C(t)_{\text{calibrator}}$$

Expression of the target gene normalized to the reference gene and relative to the calibrator  $= 2^{-\Delta\Delta C(t)}$

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

## 8. Troubleshooting

<b>No increase in fluorescence signal</b>	
<b>Possible causes</b>	<b>Comments and suggestions</b>
Error in cycler setup	<ul style="list-style-type: none"> <li>• Check that instrument settings correspond with the experiment.</li> </ul>
Missing components (e.g. primers or template) or pipetting error	<ul style="list-style-type: none"> <li>• Check the assembly of the reactions.</li> <li>• Check the concentrations and storage conditions of the reagents.</li> </ul>
Missing essential step in the cycling protocols	<ul style="list-style-type: none"> <li>• Check the cycling protocol (see page 9).</li> </ul>
qPCR primer design or concentration not optimal	<ul style="list-style-type: none"> <li>• Check primer design. See page 4, PCR primers.</li> <li>• Use primer concentration of 0.3-1.0 <math>\mu\text{M}</math>.</li> </ul>
Sample not configured properly in the cycler software	<ul style="list-style-type: none"> <li>• Check the plate configuration fed into the cycler software.</li> </ul>
<b>Late increase in fluorescence signal</b>	
<b>Possible causes</b>	<b>Comments and suggestions</b>
Error in cycler setup	<ul style="list-style-type: none"> <li>• Check that instrument settings correspond with the experiment.</li> </ul>
Missing components or pipetting error	<ul style="list-style-type: none"> <li>• Check the assembly of the reactions.</li> <li>• Check the concentrations and storage conditions of the reagents.</li> </ul>
Insufficient activation of the hot start DNA polymerase	<ul style="list-style-type: none"> <li>• Make sure the initial reactivation / denaturation step was at least 7 min at 95 °C.</li> <li>• Make sure the cycler is properly calibrated to ensure that the block temperature is accurate.</li> </ul>
Insufficient starting template	<ul style="list-style-type: none"> <li>• Increase template amount if possible.</li> </ul>
qPCR primer design not optimal	<ul style="list-style-type: none"> <li>• Check primer design. See page 4, PCR primers.</li> </ul>
qPCR primer concentration too low	<ul style="list-style-type: none"> <li>• Increase qPCR primer concentration (to max 1 <math>\mu\text{M}</math> each).</li> </ul>
Annealing temperature too high	<ul style="list-style-type: none"> <li>• Use the cycler's gradient feature (if available) to optimize annealing temperature.</li> <li>• Decrease annealing temperature in 2 °C decrements if a gradient feature is not available.</li> </ul>
Insufficient extension time for the amplicon size	<ul style="list-style-type: none"> <li>• We recommend 15–30 s extension time for &lt;250 bp amplicons.</li> <li>• Increase extension time up to 30 s.</li> </ul>
Cycling protocol not optimal	<ul style="list-style-type: none"> <li>• Use cycling protocol shown in table 4. If necessary, optimize using the protocol in table 4 as a starting point.</li> </ul>

<b>Normal fluorescence signal, but melting curve analysis shows primer-dimers or nonspecific products only</b>	
<b>Possible causes</b>	<b>Comments and suggestions</b>
Missing components or pipetting error	<ul style="list-style-type: none"> <li>• Check the assembly of the reactions.</li> <li>• Check the concentrations and storage conditions of the reagents.</li> </ul>
Primer–dimers from a previous run contaminating the reaction	<ul style="list-style-type: none"> <li>• Perform UNG treatment prior to PCR cycling.</li> </ul>
Annealing temperature of qPCR primers too low	<ul style="list-style-type: none"> <li>• Use the cycler's gradient feature (if available) to optimize annealing temperature.</li> <li>• Decrease annealing temperature in 2 °C decrements if a gradient feature is not available.</li> </ul>
qPCR primer design not optimal	<ul style="list-style-type: none"> <li>• Check primer design. See page 4, PCR primers.</li> </ul>
<b>Normal fluorescence signal, melting curve analysis shows both primer-dimer or nonspecific product and specific product peaks</b>	
<b>Possible causes</b>	<b>Comments and suggestions</b>
Low initial template concentration	<ul style="list-style-type: none"> <li>• Increase template amount.</li> </ul>
qPCR primer design not optimal	<ul style="list-style-type: none"> <li>• Check primer design. See page 4, PCR primers.</li> </ul>
Primer concentration too high	<ul style="list-style-type: none"> <li>• Optimize primer concentration by titrating between 0.3 and 1 µM.</li> </ul>
Annealing temperature of qPCR primers too low	<ul style="list-style-type: none"> <li>• Use the cycler's gradient feature (if available) to optimize annealing temperature.</li> <li>• Decrease annealing temperature in 2 °C decrements if a gradient feature is not available.</li> </ul>
Primer–dimers or PCR products from previous run contaminating the reaction	<ul style="list-style-type: none"> <li>• Perform UNG treatment prior to PCR cycling.</li> </ul>
Co-amplification of primer-dimers with the specific product	<ul style="list-style-type: none"> <li>• Perform a second data acquisition at an elevated temperature to minimize the interference of primer-dimers (see pages 9 and 11).</li> </ul>
Extension time in qPCR too long	<ul style="list-style-type: none"> <li>• Decrease extension time.</li> </ul>
<b>Non-linear correlation between C(t) and log of template amount in the standard curve</b>	
<b>Possible causes</b>	<b>Comments and suggestions</b>
Template dilution inaccurate	<ul style="list-style-type: none"> <li>• Remake dilution series and make sure the samples are well mixed.</li> <li>• Make sure that the pipettes are calibrated.</li> </ul>
Template amount too high	<ul style="list-style-type: none"> <li>• Reduce the template amount.</li> </ul>
Template amount too low	<ul style="list-style-type: none"> <li>• Increase template amount.</li> </ul>
Insufficient activation of the hot start DNA polymerase	<ul style="list-style-type: none"> <li>• Make sure the initial reactivation / denaturation step was at least 7 min at 95 °C.</li> <li>• Make sure the cycler is properly calibrated to ensure that the block temperature is accurate.</li> </ul>

Co-amplification of primer-dimers with the specific product	<ul style="list-style-type: none"> <li>Perform a second data acquisition at an elevated temperature to minimize the interference of primer-dimers.</li> </ul>
qPCR primer design or concentration not optimal	<ul style="list-style-type: none"> <li>Check primer design. See page 4, PCR primers.</li> <li>Use primer concentration of 0.3-1.0 <math>\mu</math>M.</li> </ul>
<b>High initial fluorescence signal, which gradually decreases over the first 10-20 cycles</b>	
<b>Possible causes</b>	<b>Comments and suggestions</b>
Template amount too high	<ul style="list-style-type: none"> <li>Reduce the template amount.</li> </ul>
Insufficient denaturation of template	<ul style="list-style-type: none"> <li>Make sure the initial reactivation / denaturation step was at least 7 min at 95 °C.</li> <li>Make sure the cyclor is properly calibrated to ensure that the block temperature is accurate.</li> </ul>

## Appendix I: General molecular biology data

**Table 1. Spectrophotometric conversions for nucleic acid templates.**

1 A <sub>260</sub> unit*	Concentration ( $\mu$ g/ml)
Double-stranded DNA	50
Single-stranded DNA	33
Single-stranded RNA	40

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

**Table 2. Molar conversions for nucleic acid templates.**

Nucleic acid	Size	pmol/ $\mu$ g	Copies/ $\mu$ g**
1 kb DNA	1 000 bp	1.52	$9.1 \times 10^{11}$
pUC19DNA	2 686 bp	0.57	$3.4 \times 10^{11}$
Lambda DNA	48 502 bp	0.03	$1.8 \times 10^{10}$
<i>Escherichia coli</i>	$4.7 \times 10^6$ bp	$3.2 \times 10^{-4}$	$1.9 \times 10^8$
Human	$3.2 \times 10^9$ bp	$4.7 \times 10^{-7}$	$2.8 \times 10^5$

\*\* For single-copy genes.

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## Ordering information

- F-415S** 40 reactions (50 µl each) or 100 reactions (20 µl each)
- F-415L** 200 reactions (50 µl each) or 500 reactions (20 µl each)
- F-415XL** 1000 reactions (50 µl each) or 2500 reactions (20 µl each)

When using RNA as starting material, we recommend DyNAmo™ cDNA synthesis Kit (F-470) for producing cDNA.

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