

# DyNAmo™

## SYBR® Green qPCR Kits

### Instruction manual

- F- 400S** DyNAmo™ SYBR® Green qPCR Kit, sufficient for 40 reactions (50 µl each)
- F- 400L** DyNAmo™ SYBR® Green qPCR Kit, sufficient for 200 reactions (50 µl each)
  
- F- 400RS** DyNAmo™ SYBR® Green qPCR Kit with ROX™ passive reference dye, sufficient for 40 reactions (50 µl each)
- F- 400RL** DyNAmo™ SYBR® Green qPCR Kit with ROX™ passive reference dye, sufficient for 200 reactions (50 µl each)

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# Description

Finnzymes' DyNAmo™ SYBR® Green qPCR Kits are a very sensitive and easy to use solution 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. The performance of DyNAmo SYBR Green qPCR Kits is based on a modified *Thermus brockianus* DNA polymerase, which has been specifically engineered to provide superior detection of low copy targets and shorter overall qPCR times. A non-specific DNA binding domain has been fused to the *Tbr* DNA polymerase. The domain lends physical stability to the polymerase DNA complex. SYBR Green I is specific for double-stranded DNA and fluoresces when bound to the amplified double-stranded PCR product, thereby enabling the direct quantitation of amplified DNA without labeled probes.

The reaction chemistry of DyNAmo SYBR Green qPCR Kits is applicable to most block-based real-time qPCR 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-420S, F-420L). If you use RNA as your starting material, we recommend you to use DyNAmo SYBR Green 2-Step qRT-PCR Kits for block-based (F-430S/L) and for capillary-based (F-440S/L) instruments.

# Applications

- Profiling gene expression
- Detection and quantification of RNA or DNA targets
- Microbial detection
- Viral load determination

## Kit Components

DyNAmo™ SYBR® Green qPCR Kkit	F-400S	F-400L
2x master mix (contains 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 20 µl)
DyNAmo™ SYBR® Green qPCR Kit with ROX passive reference dye	F-400RS	F-400RL
2x master mix (contains 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 20 µl)
50x ROX passive reference dye	1 x 50 µl	1 x 250 µl

\*Provides 2.5 mM MgCl<sub>2</sub> in 1x reaction concentration.

## Shipping and Storage

DyNAmo qPCR Kits are shipped in gel ice. Upon arrival, store all kit components at +4 °C or 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 one year at -20 °C and six months at +4 °C from the date of packaging when stored and handled properly.

## General Considerations

**Table 1. General recommendations**

Categories	Comments
Kit storage	Store at +4 °C or -20 °C.
Consumables	Follow the recommendations of the PCR instrument manufacturer.
Reaction volume	20 - 50 µl
Amplicon size	< 500 bp
Template amount	Depends on template type and quality. In general do not use more than 500 ng genomic DNA in a 50 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> , <i>Proc. Nat. Acad. Sci.</i> 83, 3746-50 (1986).) 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> . Can be optimized up to 5mM.

## Reaction volume

Reaction volume from 20 to 50  $\mu$ l is recommended for most real-time instruments. When >500 ng of DNA template is used, a larger reaction volume is recommended (up to 100  $\mu$ l).

## 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.

## DNA polymerase

The 2x qPCR master mix in DyNAmo SYBR Green qPCR Kits include a modified *Thermus brockianus* DNA polymerase. The modified polymerase incorporates a non-specific DNA binding domain that lends physical stability to the polymerase-DNA complex.

## Standards

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 amount of DNA. (C(t) = cycle threshold, the cycle number at which the fluorescence signal reaches the threshold level. 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 standard and target molecules.

## 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.*, Gene 93, 125-28 (1990)). UNG is inactivated during the first denaturation step in PCR. 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 which is used to set up reactions.

## 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. In most cases, good results are obtained using a concentration of 0.3  $\mu\text{M}$  for each primer. The optimum primer concentration is usually between 0.1 and 1  $\mu\text{M}$ .

## MgCl<sub>2</sub> optimization

Generally, it is not necessary to optimize the MgCl<sub>2</sub> concentration with DyNAmo SYBR Green qPCR Kits. For most reactions, we recommend a final concentration of 2.5 mM MgCl<sub>2</sub>, as provided in the master mix. However, in some cases improved results may be obtained with higher MgCl<sub>2</sub> concentrations. Excessive MgCl<sub>2</sub> concentrations can lead to the amplification of non-specific products and primer-dimers. Usually no more than 5 mM MgCl<sub>2</sub> is required by any amplicon.

## ROX™ passive reference dye (included in kits F-400RS/L)

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 the excitation. Real-time cyclers that use argon laser as excitation light source or have excitation filters not optimal for ROX dye might need more 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. At 1x working concentration the ROX dye can be used to normalize data with ABI real-time PCR instruments e.g. 7700 and 7900 SDS models. In ABI 7500 where excitation light is filtered more optimally for ROX dye, lower amount of ROX dye is sufficient. ROX dye concentration optimum is usually between 0.03 - 1x concentration. Add the ROX passive reference dye to the qPCR master mix, 0.03 - 1  $\mu\text{l}$  for each 50  $\mu\text{l}$  reaction. Dilute ROX dye in ddH<sub>2</sub>O before pipetting if necessary.

## **qRT-PCR**

To determine the quantity of an mRNA, a reverse transcription (RT) reaction must be performed prior to qPCR. Commercial cDNA synthesis kits are available for this purpose. Finnzymes offers DyNAmo SYBR Green 2-Step qRT-PCR Kits for block-based (F-430S/L) and for capillary-based (F-440S/L) instruments. Alternatively, stand-alone enzymes may be used, but RT efficiency must be checked for each experiment.

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 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**

Specific primers, random hexamers or oligo(dT) primers can be used for the RT step. Using specific primers can decrease background, whereas 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**

qRT-PCR primers should be designed to anneal to sequences in two exons on the opposite sides of an intron to differentiate between amplification of cDNA and contaminating genomic DNA. An amplification product from genomic DNA will be larger than the mRNA derived product that lacks the intron sequence. Alternatively, primers can be designed to anneal to the exon-exon boundary of the mRNA. With such primers the amplification of genomic DNA will be highly inefficient.

## **DNase I**

If primers cannot be designed in the exon / exon boundaries or in separate exons, it is necessary to treat the RNA sample 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 (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. A reference gene or several genes are used to normalize data against variation in sample quality and quantity. 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 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.

The amplification efficiency of a reference gene should be the same as the efficiency of the target gene. If this is not the case, the results have to be corrected for the efficiency.

# Reaction Setup

- Perform the reaction setup in an area separate from nucleic acid preparation or PCR product analysis.
- Pipet all components on ice.
- Pipet with sterile filter tips.
- Minimize the exposure of the 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 maximal signal.
- Use a cap sealing tool or firm finger pressure to properly close caps or film sealer.
- Avoid touching the optical surface of the cap or sealing film without gloves, as fingerprints may interfere with fluorescence measurements.
- Plates or strips should be centrifuged before starting the cycling program to force the solution to the bottom of the tube and to remove any possible bubbles.

## Protocol

1. Program the cycler as outlined in table 3.
2. Thaw template DNA, primers and master mix (and ROX passive reference dye). Mix the individual solutions to assure homogeneity (this is especially important for the master mix) and keep on ice.
3. Prepare a PCR premix by mixing master mix, primers, (ROX passive reference dye if using kits F-400RS/L) and ddH<sub>2</sub>O (see table 2). Mix the PCR premix thoroughly to assure homogeneity. Dispense appropriate volumes into strip tubes or plate wells and store on ice.
4. Add template DNA (< 500 ng / 50 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.
5. Place the strips or plate in the thermal cycler and start the cycling program.

**Table 2. Reaction setup.**

Components (In order of addition)	Stock	Volume / 50 µl reaction	Volume / 20 µl reaction	Final conc.	Comments
Master mix	2x	25 µl	10 µl	1x	Mix thoroughly.
Primer mix (in ddH <sub>2</sub> O)		X µl	X µl	0.3 µM fwd 0.3 µM rev	Titrate from 0.1 to 1 µM if necessary.
ROX reference dye	50x	(0.03 - 1 µl)	(0.012 - 0.4 µl)	0.03 - 1x	Optional (included in kits F-400RS/L. See page 5)
Template DNA (in ddH <sub>2</sub> O)		X µl	X µl		Do not exceed 10 ng/µl in the final reaction.
ddH <sub>2</sub> O		X µl	X µl		Add water to fill up to the final reaction volume.
<b>Total volume</b>		<b>50 µl</b>	<b>20 µl</b>		

For different volumes, adjust all components proportionally.

# Cycling Protocol

Table 3. Cycler protocol.

Step	Purpose	Temp	Time	Comments
1	Block preheating	95 °C or 50 °C	Indefinite	This step is optional, but recommended if possible. If UNG is used, pre-incubate at 50 °C. After inserting reaction strips or plate into the cycler proceed to next step.
1b*	UNG incubation (optional)	50 °C	2 min	If heat labile UNG is used, lower the incubation temperature and increase time according to manufacturer's instructions.
2	Initial denaturation	95 °C	10 min	Shorter time can be sufficient when short template is used and total DNA amount is low.
3	Denaturation	94 °C	10 s	
4**	Annealing	X °C	10-20 s	5 °C below lower primer T <sub>m</sub> , use gradient feature to optimize.
5	Extension	72 °C	5-20 s or 4 s/100 bp	Use shortest time possible for best results.
6	Data acquisition			Fluorescence data collection.
7***	Optional	X °C	1 s	T <sub>m</sub> (primer-dimer) < X < T <sub>m</sub> (product)
8***	Data acquisition (optional)			Fluorescence data collection at higher than extension temperature prevents errors in case primer-dimers are observed.
9	Number of cycles	35-45 cycles, steps 3-8		
10	Final extension (optional)	72 °C	5-10 min	Final extension ensures that all amplification products are in a double-stranded form before melting curve step.
11	Melting curve	72-95 °C	20 min ramp time for most instruments.	Note that melting curve setting options vary between different real-time instruments. See instrument manufacturer's manuals for detailed information.
12	Reannealing	72 °C	5-10 min	Reannealing is important if the samples are to be analysed on an agarose gel.

\* Step 1b can be excluded if UNG is not used.

\*\* Use the nearest-neighbor method (Breslauer *et al.*, *Proc. Nat. Acad. Sci.* 83, 3746-50 (1986)) to determine T<sub>m</sub> of the primers. Use 50 mM KCl and 300 nM primer concentration when calculating T<sub>m</sub> (or the primer concentration in your reaction if optimized to other than 300 nM). Due to the characteristics of the modified DNA polymerase it is often possible to use higher annealing temperatures than with other enzymes.

\*\*\* Steps 7 and 8 are to be used if significant amounts of primer-dimers are co-amplified with the specific product (Morrison, T.B., *et al.*, *Biotechniques* 24, 954-62 (1998)).

## Cycling steps

### Block preheating

Block preheating (step 1) before inserting reactions into the cycler reduces the risk of primer-dimer formation.

### UNG incubation

If UNG enzyme is used, incubate 2 min at 50 °C (step 1b). If heat labile UNG is used, lower the incubation temperature and increase time according to manufacturer's instructions.

### Initial denaturation

Initial denaturation (step 2) at 95 °C for 10 min is sufficient in most cases to assure that the starting template is denatured. As short as 1-minute denaturation can be sufficient when a short template is used and the amount of total DNA is low. If UNG is used, use a denaturation time sufficient to inactivate UNG enzyme, typically 3 min.

### Denaturation

Denaturation (step 3) at 94 °C for 10 s is sufficient in most cases.

### Annealing

For most amplicons, annealing (step 4) for 20 s at 5 °C below the lower T<sub>m</sub> of the two primers works well as a starting point. In many cases, 60 °C can be used with success for a wide range of primer pairs. Due to the unique characteristics of the modified *Tbr* 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.

These guidelines are based on T<sub>m</sub> values calculated (50 mM salt and 300 nM primer) with the nearest-neighbor method as described by Breslauer *et al.*, *Proc. Nat. Acad. Sci.* 83, 3746-50 (1986). Different software may give different T<sub>m</sub> values. A temperature gradient may be used to find the optimal annealing temperature for each starting template-primer pair combination. If primer-dimers are still observed, the reaction conditions may be further optimized by reducing the primer concentrations and using shorter annealing time. Instructions for T<sub>m</sub> 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)).

### Extension

Extension temperature (step 5) should be 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. A combined annealing / extension step also can be used for developing two-step protocols. Extension time depends on amplicons length, and should be as short as possible. For amplicons <500 bp, 30 s extension time generally gives good results.

## **Data acquisition**

If primer-dimers are observed, it may be helpful to perform a data acquisition step at an elevated temperature (steps 7&8) 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 should be sufficient for most applications even when the template is present at 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 10) 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 11).

## **Melting curve**

A melting curve (step 11) 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 non-specific products by the difference in their melting temperatures. The recommended temperature ramp rate is stated in table 3. If a faster protocol is preferred, the ramp rate of the melting curve can be increased, however 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  $72\text{ }^\circ\text{C}$  to for example  $65\text{ }^\circ\text{C}$ . In that case, the final extension (step 10) should be also be performed at the same temperature.

## **Reannealing**

The reannealing step (step 12) is recommended if agarose gel analysis is to be performed on the final products. This allows the reformation of fully duplexed DNA.

# **Analysis**

## **Melting curve**

Melting curve analysis is typically included in the analysis software of real-time fluorescent 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  $T_m$ ,

or the melting temperature of the product. If primer-dimers or other non-specific products are observed, the efficiency of the PCR should be checked. Varying efficiency leads to incorrect quantification.

## Absolute quantification

Absolute quantification 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.

## Relative quantification

Relative quantification 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 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 quantification. 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 =  $2^{-\Delta\Delta C(t)}$ .

# 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 cycler protocols	<ul style="list-style-type: none"> <li>• Check the cycler protocol.</li> </ul>
qPCR primer design or concentration not optimal	<ul style="list-style-type: none"> <li>• Re-check primer design. See page 5, PCR primers.</li> <li>• Use primer concentration of 0.1-1.0 <math>\mu\text{M}</math>.</li> </ul>
Sample not configured properly	<ul style="list-style-type: none"> <li>• Check the plate configuration.</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 starting template	<ul style="list-style-type: none"> <li>• Check the calculation of template stock concentration; increase template amount if possible.</li> </ul>
qPCR primer design not optimal	<ul style="list-style-type: none"> <li>• Re-check primer design. See page 5, 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 gradient to optimize annealing temperature.</li> <li>• Decrease annealing temperature in 2 <math>^{\circ}\text{C}</math> decrements if a gradient feature is not available.</li> </ul>
Insufficient extension time for the amplicon size	<ul style="list-style-type: none"> <li>• Use &gt; 20 s if the amplicon is &gt;500 bp and/or has a high GC content.</li> </ul>
PCR protocol not optimal	<ul style="list-style-type: none"> <li>• Make sure the recommended PCR protocol is used. If necessary, optimize using the recommended protocol as a starting point.</li> </ul>
<b>Normal fluorescence signal, but melting curve analysis shows primer-dimers or non-specific 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 gradient to optimize annealing temperature.</li> <li>• Increase annealing temperature in 2 <math>^{\circ}\text{C}</math> increments if a gradient feature is not available.</li> </ul>
qPCR primer design not optimal	<ul style="list-style-type: none"> <li>• Re-check primer design. See page 5, PCR primers.</li> </ul>

<b>Normal fluorescence signal, melting curve analysis shows both primer-dimer or non-specific 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>• Re-check primer design.</li> </ul>
Primer concentration too high	<ul style="list-style-type: none"> <li>• Optimize primer concentration. Titrate from 0.1 to 1 <math>\mu\text{M}</math>.</li> </ul>
Annealing temperature of qPCR primers too low	<ul style="list-style-type: none"> <li>• Use gradient to optimize annealing temperature.</li> <li>• Increase annealing temperature in 2 <math>^{\circ}\text{C}</math> increments 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.</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> </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>
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>• Re-check primer design. See page 5, PCR primers.</li> <li>• Use primer concentration of 0.1-1.0 <math>\mu\text{M}</math>.</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>• Increase the length of the initial denaturation step (95 <math>^{\circ}\text{C}</math> up to 10 min).</li> <li>• Make sure cycler 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 (µg/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/µg	Copies/µg**
1 kb DNA	1 000 bp	1.52	9.1 × 10 <sup>11</sup>
pUC19DNA	2 686 bp	0.57	3.4 × 10 <sup>11</sup>
Lambda DNA	48 502 bp	0.03	1.8 × 10 <sup>10</sup>
<i>Escherichia coli</i>	4.7 × 10 <sup>6</sup> bp	3.2 × 10 <sup>-4</sup>	1.9 × 10 <sup>8</sup>
Human	3.2 × 10 <sup>9</sup> bp	4.7 × 10 <sup>-7</sup>	2.8 × 10 <sup>5</sup>

\*\* For single-copy genes.

## Appendix II: Related Products

F-410S/L	DyNamo™ HS SYBR® Green qPCR Kit
F-420S/L	DyNamo™ Capillary SYBR® Green qPCR Kit
F-430S/L	DyNamo™ SYBR® Green 2-Step qRT-PCR Kit
F-440S/L	DyNamo™ Capillary SYBR® Green 2-Step qRT-PCR Kit
F-450S/L	DyNamo™ Probe qPCR Kit
F-460S/L	DyNamo™ Probe 2-Step qRT-PCR Kit
F-570S/L	AMV Reverse Transcriptase
F-572S/L	M-MuLV RNase H <sup>-</sup> (not available in US)

# Appendix III: Warranty

Finnzymes Oy warrants that its products will meet the specifications stated on the technical data section of the data sheets, and Finnzymes Oy agrees to replace the products free of charge if the products do not conform to the specifications. Notice for replacement must be given within 60 days of receipt. In consideration of the above commitments by Finnzymes Oy, the buyer agrees to and accepts the following conditions:

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3. That the buyer's sole remedy shall be to obtain replacement of the product free of charge from Finnzymes Oy; and
4. That this remedy is in lieu of all other remedies or claims for damages, consequential or otherwise, which the buyer may have against Finnzymes Oy.

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LightCycler is a trademark of a Member of the Roche Group.

SYBR is a registered trademark of Molecular Probes.

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## Ordering Information

- F- 400S** DyNAmo™ SYBR® Green qPCR Kit,  
sufficient for 40 reactions (50 µl each)
- F- 400L** DyNAmo™ SYBR® Green qPCR Kit,  
sufficient for 200 reactions (50 µl each)
- F- 400RS** DyNAmo™ SYBR® Green qPCR Kit *with ROX™ passive reference dye*,  
sufficient for 40 reactions (50 µl each)
- F- 400RL** DyNAmo™ SYBR® Green qPCR Kit *with ROX™ passive reference dye*,  
sufficient for 200 reactions (50 µl each)



**Finnzymes Oy**

Keilaranta 16 A

02150 ESPOO, Finland

Tel: +358-9-584 121

Fax: +358-9-5841 2200

fz@finnzymes.fi

www.finnzymes.com

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