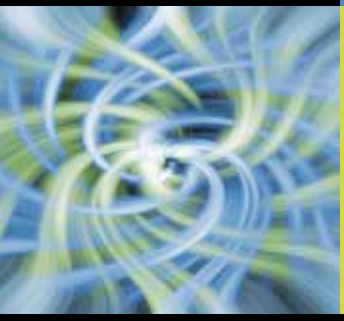

# Thermo Scientific
# DyNAmo SYBR Green qPCR Kits

## Technical Manual

| | |
|---|---|
| F- 400S | 100 reactions (20 µl each) or 40 reactions (50 µl each) |
| F- 400L | 500 reactions (20 µl each) or 200 reactions (50 µl each) |
| F- 400RS | 100 reactions (20 µl each) or 40 reactions (50 µl each) |
| F- 400RL | 500 reactions (20 µl each) or 200 reactions (50 µl each) |

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# 1. Description

Thermo Scientific DyNAmo SYBR Green qPCR Kits 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.

The performance of DyNAmo™ SYBR® Green qPCR Kits is based on a modified *Thermus brockianus* DNA polymerase and SYBR Green fluorescent dye. A non-specific DNA binding domain has been fused to the *Tbr* DNA polymerase. This 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 permitting the direct quantification 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, including those from Applied Biosystems, Bio-Rad Laboratories, Corbett Research and Stratagene. For capillary-based instruments, such as the Roche LightCycler™, we recommend Thermo Scientific DyNAmo Capillary SYBR Green qPCR Kit (F-420). When RNA is used as the starting material for producing cDNA, we recommend Thermo Scientific DyNAmo cDNA Synthesis Kit (F-470) to ensure high-quality results.

# 2. Kit components

| **DyNAmo SYBR Green qPCR Kit** | **F-400S** | **F-400L** |
|---|---|---|
| 2x master mix (contains modified *Tbr* DNA polymerase, SYBR® Green I, optimized PCR buffer, 5 mM $MgCl_2$, dNTP mix including dUTP) | 1 x 1 ml (sufficient for 100 reactions of 20 µl or 40 reactions of 50 µl) | 5 x 1 ml (sufficient for 500 reactions of 20 µl or 200 reactions of 50 µl) |
| **DyNAmo SYBR Green qPCR Kit with ROX passive reference dye** | **F-400RS** | **F-400RL** |
| 2x master mix (contains modified *Tbr* DNA polymerase, SYBR® Green I, optimized PCR buffer, 5 mM $MgCl_2$, dNTP mix including dUTP) | 1 x 1 ml (sufficient for 100 reactions of 20 µl or 40 reactions of 50 µl) | 5 x 1 ml (sufficient for 500 reactions of 20 µl or 200 reactions of 50 µl) |
| 50x ROX passive reference dye | 1 x 50 µl | 1 x 250 µl |

Material safety data sheet (MSDS) is available at www.thermoscientific.com/fzmsds.

## 3. Shipping and storage

DyNAmo qPCR Kits are shipped on 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.

## 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 | < 500 bp |
| Template amount | Depends on template type and quality. In general, do not use more than 500 ng of genomic DNA in a 50 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 $MgCl_2$, and can be optimized up to 5 mM. |

### 4.1 DNA polymerase

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

### 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 0.3 µM for each primer. The optimum primer concentration is usually between 0.1 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 (R2) 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 (included in F-400RS and F-400RL)

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 dissolved 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.

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 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 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 (uracil-N-glycosylase) before PCR. UNG 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. All Thermo Scientific 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 to set up reactions.

## 4.7 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.

## 4.8 $MgCl_2$ optimization

Generally, it is not necessary to optimize the $MgCl_2$ concentration with the DyNAmo SYBR Green qPCR Kits. For most reactions, we recommend a final concentration of 2.5 mM $MgCl_2$, as provided in the master mix. However, in some rare cases, better results may be obtained with higher $MgCl_2$ concentrations. Excessive $MgCl_2$ concentrations can lead to the amplification of nonspecific products and primer-dimers, however. Usually no more than 5 mM $MgCl_2$ is required by any amplicon.

### 4.9 Quantification of RNA

To determine the quantity of mRNA, a reverse transcription (RT) reaction must be performed before qPCR. We offer DyNAmo cDNA Synthesis Kit (F-470) for quantitative reverse transcription. DyNAmo SYBR Green qPCR Kits have been optimized using the DyNAmo cDNA Synthesis Kit.

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.
- Pipette all components 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 $H_2O$.

#### Reaction setup

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). Mix the individual solutions to ensure homogeneity. This is especially important for the master mix. Keep the solutions on ice.
3. 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, and store on ice.
4. Add template DNA (< 500 ng per 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. 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) | 50 µl reaction | 20 µl reaction | Final concentration | Comments |
|---|---|---|---|---|
| 2x master mix | 25 µl | 10 µl | 1x | Mix thoroughly. |
| Primer mix (in $H_2O$) | X µl | X µl | 0.3 µM fwd<br>0.3 µM rev | Titrate from 0.1 to 1 µM if necessary. |
| 50x ROX reference dye | (0.3–1 µl) | (0.12–0.4 µl) | 0.3–1x | Optional (see Section 4.5). |
| Template DNA | X µl | X µl | | Do not exceed 10 ng/µl in the final reaction. |
| $H_2O$ | add to 50 µl | add to 20 µl | | |

For different volumes, adjust all components proportionally.

## Cycling protocol

Table 4. Cycling protocol.

| Step | Purpose | Temp | Time | Comments |
|---|---|---|---|---|
| | Block preheating and UNG incubation | | | Optional, see below. |
| 1 | Initial denaturation | 95°C | 10 min | A shorter time can be sufficient when a short template is used and the total DNA amount is low. |
| 2 | Denaturation | 94°C | 10 s | |
| 3[1] | Annealing | X°C | 10–20 s | 5°C below lower primer Tm; use gradient feature to optimize. |
| 4 | Extension | 72°C | 5–20 s or 4 s/100 bp | For best results, use as short a time as possible. For two-step PCR, see 'Extension' on page 10. |
| 5 | Data acquisition | | | Fluorescence data collection |
| 6 | Number of cycles | 35–45 cycles, steps 3–6 | | |
| | Final extension | | | Optional, see page 11. |
| 7 | 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 the instrument manufacturer's manual for detailed information. |
| | Reannealing | | | Optional, see page 11. |

1 Use the Tm calculator at www.thermoscientific.com/pcrwebtools to determine Tm of the primers. Use 50 mM KCl and 0.3 µM primer concentration when calculating Tm (or the primer concentration in your reaction if optimized to other than 0.3 µM). Due to the characteristics of the modified DNA polymerase, it is often possible to use higher annealing temperatures than with other enzymes.

### Block preheating (optional)

Preheating the block to 95°C before inserting reactions into the cycler is optional, but recommended. It reduces the risk of primer-dimer formation. If UNG treatment is performed, only pre-heat to the UNG incubation temperature.

### UNG incubation (optional)

If UNG enzyme is used, incubate 2 minutes at 50°C. If heat-labile UNG is used, decrease the incubation temperature and increase time in accordance with the manufacturers' instructions.

### Initial denaturation / reactivation

Initial denaturation at 95°C for 10 minutes is sufficient in most cases to ensure that the starting template is denatured. A denaturation temperature as brief as 1 minute can be used if the template is short and the total DNA amount is low. However, if UNG is used, the denaturation time should be long enough to inactivate the UNG enzyme (typically 3 minutes).

### Denaturation

Denaturation at 94°C for 10 seconds is sufficient in most cases.

### Annealing

For most amplicons, annealing for 20 seconds at 5°C below the lower Tm 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* 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.

These guidelines are based on Tm values (50 mM salt and 0.3 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. Alternatively, the annealing temperature can be optimized 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.

### Extension

Extension temperature 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. The extension time depends on the amplicon length, and should be as short as possible. For amplicons < 500 bp, a 20-second extension time generally gives good results.

Depending on the amplicon a combined annealing/extension step can also be used (two-step PCR). Usually 30-60 seconds at 60°C works well for a combined annealing/extension step.

### 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 the 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 can be 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, 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.

### Reannealing (optional)

The reannealing step is recommended if agarose gel analysis is to be performed on the final products. This allows the reformation of fully duplex DNA after the melting curve step.

# 6. Analysis

## 6.1 Melting curve

Melting curve analysis is typically included in the analysis software of real-time PCR 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 making 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 = $((10^{\frac{-1}{slope}}) - 1) \times 100\ \%$
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 = $((10^{-1 \times slope}) - 1) \times 100\ \%$
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 '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 quantitation. 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:

$\Delta Cq(sample) = Cq(target) - Cq(reference)$
$\Delta Cq(calibrator) = Cq(target) - Cq(reference)$

The ΔΔCq value is then determined using the following formula:
$\Delta\Delta Cq = \Delta Cq(sample) - \Delta Cq(calibrator)$

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).

# 7. Troubleshooting

| Possible 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.<br>• 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 | • Check primer design. See Section 4.2.<br>• Use primer concentration of 0.1–1.0 µM. |
| Sample not configured properly in the cycler software | • 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.<br>• Check the concentrations and storage conditions of the reagents. |
| Template amount too low | • Check the calculation of the template stock concentration; increase the template amount if possible. |
| qPCR primer design not optimal | • 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.<br>• Decrease the annealing temperature in 2°C decrements if no gradient feature is available. |
| Insufficient extension time for the amplicon size | • If the amplicon is longer than 500 bp, or has a high GC-content, use >20 s extension time. |
| PCR protocol not optimal | • Make sure you are using the recommended PCR protocol. If necessary, optimize using the recommended protocol as a starting point. |
| **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.<br>• Check the concentrations and storage conditions of the reagents. |
| Primer-dimers from a previous run contaminating the reaction | • Perform UNG treatment before PCR cycling. |
| Annealing temperature too low | • Use gradient to optimize annealing temperature.<br>• Increase annealing temperature in 2°C increments if no gradient feature is available. |
| qPCR primer design not optimal | • 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 | • Check primer design. See Section 4.2. |
| Primer concentration too high | • Optimize primer concentration. Titrate from 0.1 to 1 µM. |
| Annealing temperature too low | • Use gradient to optimize annealing temperature.<br>• 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 UNG 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 template amount. |
| Template amount too low | • Increase template amount. |
| 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.<br>• Use primer concentration of 0.1–1.0 µM. |
| **High initial fluorescence signal, gradually decreasing over the first 10–20 cycles** | |
| Template amount too high | • Reduce template amount. |
| Insufficient denaturation of template | • Make sure 95°C 10 min is used for the initial denaturation step in qPCR.<br>• Make sure the cycler block temperature is accurate. |
| **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. |
| **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. |

* ROX passive refrence dye is included in F-400RS and F-400RL

# 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 made 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 DyNAmo cDNA Synthesis Kit (F-470) for the reverse transcription step. This kit has 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 (ΔΔCq 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 should be identical. For efficiency calculation using the slope, see Section 6.2 (Absolute quantification).

# Appendix II: general molecular biology data

Table 5. 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 6. Molar conversions for nucleic acid templates.

| Nucleic acid | Size | pmol/µg | Copies/µg* |
|---|---|---|---|
| 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 | $1.8 \times 10^{10}$ |
| *Escherichia coli* | $4.7 \times 10^{6}$ 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.

## Product use limitation

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

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ROX is a trademark of Life Technologies Corporate and its affiliated companies.
Applied Biosystems is a registered trademark of Life Technologies Corporation and its affiliated companies.
LightCycler™ is a trademark of a Member of the Roche Group.

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# Thermo Scientific DyNAmo SYBR Green qPCR Kits
# Technical Manual



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