



Thermo Scientific DyNAmo Probe qPCR Kit

Technical Manual

- F- 450S 100 reactions (20 μ l each) or 40 reactions (50 μ l each)
- F- 450L 500 reactions (20 μ l each) 200 reactions (50 μ l each)
- F- 450XL 2500 reactions (20 μ l) each or 1000 reactions (50 μ l each)

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1. Description

Thermo Scientific DyNAmo Probe qPCR Kit is designed for quantitative, real-time analysis of DNA samples from various sources using probe-based detection. Quantitative PCR (qPCR) is a useful technique for the investigation of gene expression, viral load, pathogen detection, and numerous other applications.

The performance of the DyNAmo™ Probe qPCR Kit is based on a hot-start *Thermus brockianus* (*Tbr*) DNA polymerase. *Tbr* DNA polymerase is chemically engineered to be inactive at room temperature. The inactivation prevents the extension of nonspecifically bound primers during reaction setup and therefore increases PCR specificity. Reactions can be set up at room temperature. The initial denaturation step in the PCR protocol reactivates the polymerase (hot start).

The reaction chemistry of DyNAmo Probe qPCR Kit is applicable to most block-based and capillary-based real-time PCR instruments, including those from Applied Biosystems, Roche, Bio-Rad Laboratories, Corbett Research, and Stratagene. When RNA is used as a starting material, we recommend Thermo Scientific DyNAmo cDNA Synthesis Kit (F-470). For faster protocols, Thermo Scientific DyNAmo Flash Probe qPCR Kits (F-455 and F-456) are available. For a full selection of products for both SYBR® Green and probe-based detection, go to www.thermoscientific.com/qpcrsolutions.

2. Kit components

| DyNAmo Probe qPCR Kit | F-450S | F-450L | F-450XL |
|---|--|---|--|
| 2x master mix (contains hot-start <i>Tbr</i> DNA polymerase, optimized PCR buffer, 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) | 25 x 1 ml (sufficient for 2500 reactions of 20 µl or 1000 reactions of 50 µl) |
| 50x ROX passive reference dye | 1 x 50 µl | 1 x 250 µl | 1 x 1.25 ml |

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

3. Shipping and storage

The DyNAmo Probe 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.

4. Notes about reaction components

Table 1. General recommendations.

| Categories | Comments |
|-----------------|--|
| Kit storage | Store at -20°C. |
| Consumables | Follow the recommendations of the PCR instrument manufacturer. |
| Reaction volume | 20–50 µl |
| Amplicon size | < 250 bp |
| Template amount | Depends on template type and quality. In general, do not use more than 500 ng of genomic DNA in a 50 µl reaction. |
| Primer design | Use primers with matched T _m . Avoid inter-primer and intra-primer complementary sequences. We recommend calculating T _m by 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 _m calculation and a link to a calculator using a modified nearest-neighbor method can be found on the Thermo Scientific website (www.thermoscientific.com/pcrwebtools). |

4.1 Hot start *Tbr* DNA polymerase

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

4.2 Probe-based detection chemistries

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

Hydrolysis probes (TaqMan®, Double Dye, etc.)

Hydrolysis probe chemistry is the most widely used probe-based chemistry in real-time PCR. A hydrolysis probe consists of a target specific sequence, which is usually around 20 bp long. The probe has a fluorescent reporter molecule (fluorophore) in one end and a quencher in the other end of the probe. The quencher receives the energy from the fluorophore and quenches the fluorescence. During the PCR protocol the probe hybridizes to its complementary sequence in the target and one of the PCR primers anneals in the same strand close upstream from the probe. When the polymerase extends the primer it

encounters the probe, hydrolyses it from the 5' end, and thus cleaves the reporter from the probe. When the reporter is cleaved it is no more quenched and the increase in the fluorescence can be measured with the real-time PCR instrument.

4.3 PCR primers and probes

Careful primer and probe design is important to minimize nonspecific primer annealing and primer-dimer formation. Standard precautions must be taken to avoid primer-dimer or hairpin loop formation. Many software tools for designing PCR primers and probes simultaneously are available.

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

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

4.4 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.

4.5 Standards

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

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

4.6 ROX™ passive reference dye

For most real-time instruments ROX™ passive reference dye is not required, but on some instruments it is used to normalize for non-PCR-related fluorescence signal variation. Passive reference dye does not take part in the PCR reaction and its fluorescence remains constant during the PCR reaction. The amount of ROX passive reference dye needed can vary depending on the type of excitation. The amount of ROX dye needed with real-time cyclers which use argon laser as the excitation light source or which have excitation filters that are not optimal for ROX dye may be greater than with instruments that excite efficiently near 585 nm.

The ROX dye is provided as a 50x solution 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) |

4.7 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. The UNG treatment step (50°C for 2 min) has no negative effect on qPCR performance because the hot-start *Tbr* DNA polymerase is not reactivated at 50°C. All Thermo Scientific DyNAmo qPCR Kits contain dUTP and therefore UNG treatment can be used.

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

4.8 Reaction volume

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

4.9 Multiplex qPCR

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

4.10 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 producing cDNA, or alternatively, DyNAmo Probe 2-Step qRT-PCR Kit (F-560). DyNAmo Probe qPCR Kit has 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.
- As the hot-start *Tbr* DNA polymerase is inactive during PCR setup, it is not necessary to do the setup on ice.
- Make sure all the reaction components are properly mixed.
- Pipette with sterile filter tips.
- Minimize the exposure to light after adding ROX passive reference dye and/or probe to the 2x master mix.
- Minimize pipetting errors by using calibrated pipettes and by preparing premixes to avoid pipetting very small volumes.
- Use optically clear caps or sealers to achieve maximum signal.
- Use a cap sealing tool or firm finger pressure to close caps properly, or use a film sealer.
- Avoid touching the optical surface of the cap or sealing film without gloves, as fingerprints may interfere with fluorescence measurements.
- Use powder-free gloves.
- Plates or strips should be centrifuged before starting the cycling program to force the solution to the bottom of the tubes and to remove any bubbles.
- Use molecular biology grade H_2O .

5.1 General protocol for all instruments

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

Reaction setup

1. Program the cycler as outlined in Table 4.
2. Thaw the template DNA, primers, probe and master mix (and the ROX passive reference dye, if necessary). Mix the individual solutions to ensure homogeneity. This is especially important for the master mix.
3. Prepare a PCR premix by mixing the master mix, primers, probe, (ROX if used,) and H₂O. Mix the PCR premix thoroughly to ensure homogeneity. Dispense appropriate volumes into strip tubes or plate wells.
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 for Hydrolysis probes (TaqMan®, Double Dye, etc.).

| Components (In order of addition) | 50 µl reaction | 20 µl reaction | Final concentration | Comments |
|--------------------------------------|-------------------|-------------------|---------------------------|---|
| 2x master mix | 25 µl | 10 µl | 1x | Mix thoroughly. Avoid air bubble formation. |
| Primer mix (in H ₂ O) | X µl | X µl | 0.5 µM fwd 0.5 µM rev | Titrate from 0.05 to 1 µM if necessary. |
| Probe | X µl | X µl | 0.25 µM (TaqMan probe) | Titrate from 0.05 to 0.5 µM if necessary |
| 50x ROX reference dye | (0.03–1 µl) | (0.012–0.4 µl) | 0.03–1x | Optional (see Section 4.6 and 5.2). |
| Template DNA | X µl | X µl | | Do not exceed 10 ng/µl in the final reaction. |
| H ₂ O | add to 50 µl | add to 20 µl | | |

For different volumes, adjust all components proportionally.

Cycling protocol

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

| Step | Purpose | Temp | Time | Comments |
|----------------|-----------------------|-------------------------|--------|---|
| | UNG incubation | | | Optional, see below. |
| 1 | Initial denaturation | 95°C | 15 min | This step is needed to activate the hot start <i>Tbr</i> DNA polymerase and to denature the template DNA. |
| 2 | Denaturation | 95°C | 15 s | |
| 3 ¹ | Annealing + extension | 60°C | 60 s | Temperature and time can be adjusted, but it is important that the polymerase and exonuclease activities are both functional at this step and the probe is hybridized to the target sequence. |
| 4 | Data acquisition | | | Fluorescence data collection |
| 5 | Number of cycles | 35–45 cycles, steps 2–4 | | |

1 Use the T_m calculator at www.thermoscientific.com/pcrwebtools to determine T_m of the primers. Use 50 mM KCl and 0.5 μ M primer concentration when calculating T_m (or the primer concentration in your reaction if optimized to other than 0,5 μ M). Design primers to anneal efficiently at 60°C (T_m should be about 65°C).

UNG incubation (optional)

If UNG enzyme is used, incubate 2 min at 50°C. This step does not negatively affect qPCR performance because the hot-start DNA polymerase is not active at 50°C. If heat-labile 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 15 min is needed to ensure a complete reactivation of the hot-start DNA polymerase and denaturation of the template.

Denaturation

Denaturation at 95°C for 15 s is sufficient in most cases.

Annealing

For most amplicons, a combined annealing and extension for 60 seconds at 60°C works well if the primers are designed to anneal efficiently at 60°C (T_m about 65°C). An annealing temperature of 60°C has proven successful with a wide range of primer pairs.

These guidelines are based on T_m values (50 mM salt and 0.5 μ M primer) calculated by the nearest-neighbor method as described by Breslauer *et al.* (1986) *Proc. Nat. Acad. Sci.* 83: 3746–50. Instructions for T_m calculation and a link to a calculator using a modified nearest-

neighbor method can be found on the Thermo Scientific website (www.thermoscientific.com/pcrwebtools). Different software may give different T_m values.

If needed, 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.

Number of cycles

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

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

Addition of ROX™ passive reference dye

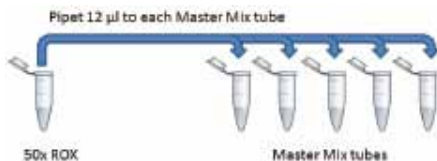
ABI 7000, 7300, 7700, 7900 and StepOne™: 1x ROX final concentration

1. Thaw and carefully mix 50x ROX and 2x Master mix tubes.
2. Add 40 µl of 50x ROX to each 1 ml 2x Master mix tube.
3. Mix again carefully.
4. Store at -20°C.



ABI 7500: 0.3x ROX final concentration

1. Thaw and carefully mix 50x ROX and 2x Master mix tubes.
2. Add 12 µl of 50x ROX to each 1 ml 2x Master mix tube.
3. Mix again carefully.
4. Store at -20°C.



Reaction setup for all Applied Biosystems models:

1. Program the cycler as outlined in Table 6.
2. Thaw template DNA, primers, probe(s) and 2x Master mix (where ROX passive reference dye has been added). Mix the individual solutions to assure homogeneity. This is especially important for the Master mix.
3. Prepare a PCR premix by mixing 2x Master mix, primers, probe(s), and H₂O. Mix the PCR premix thoroughly to ensure homogeneity. Dispense appropriate volumes into strip tubes or plate wells. Use reverse pipeting technique to avoid bubbles.
4. Add template DNA (<200 ng/20 µl reaction) to the strip tubes or plate wells containing the PCR premix. For two-step qRT-PCR, the volume of the cDNA added (from the RT reaction) should not exceed 10 % of the final PCR volume.
5. Seal the strips or plate with appropriate sealer, place them in the thermal cycler and start the cycling program.

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

| Components (In order of addition) | 50 µl reaction | 20 µl reaction | Final concentration | Comments |
|---|----------------|----------------|----------------------------|---|
| 2x Master mix with ROX added (see instructions above) | 25 µl | 10 µl | 1x | Mix thoroughly. Avoid air bubble formation. |
| Primer mix (in H ₂ O) | X µl | X µl | 0.3 µM fwd 0.3 µM rev | Titrate from 0.05 to 1 µM, if necessary |
| Probe | X µl | X µl | 0.25 µM (TaqMan® probe) | Titrate from 0.05 to 0.5 µM, if necessary. |
| Template DNA (in H ₂ O) | X µl | X µl | | Do not exceed 10 ng/µl in the final reaction. |

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

| Step | Temp. | Time | Cycles |
|----------------------|-------|--------|-----------|
| Initial denaturation | 95°C | 15 min | 1 |
| Denaturation | 95°C | 15 s | 40 cycles |
| Annealing/extension | 60°C | 60 s | |

6. Analysis

6.1 Absolute quantification

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

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

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

A slope of -3.322 corresponds to 100 % efficiency.

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

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

A slope of -0.301 corresponds to 100 % efficiency.

6.2 Relative quantification

Relative quantification is used to determine the ratio between the quantity of a target molecule in a sample and in the calibrator (healthy tissue or untreated cells, for example). The most common application of this method is the analysis of gene expression, such as comparisons of gene expression levels in different samples, for example. The target molecule quantity is usually normalized with a reference gene (see chapter 'Reference genes' in Appendix I: cDNA synthesis).

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

$$\Delta Cq(\text{sample}) = Cq(\text{target}) - Cq(\text{reference})$$

$$\Delta Cq(\text{calibrator}) = Cq(\text{target}) - Cq(\text{reference})$$

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

$$\Delta\Delta Cq = \Delta Cq(\text{sample}) - \Delta Cq(\text{calibrator})$$

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 the efficiency 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 | <ul style="list-style-type: none"> • Make sure that the instrument settings are correct for the experiment. |
| Missing components (e.g. primers, probe or template) or pipetting error | <ul style="list-style-type: none"> • Check the assembly of the reactions. • Check the concentrations and storage conditions of the reagents. |
| Missing essential step in the cycler protocol | <ul style="list-style-type: none"> • Check the cycler protocol. |
| qPCR primer and/or probe design or concentration not optimal | <ul style="list-style-type: none"> • Re-check primer and probe design. See Section 4.3. • Use primer concentration of 0.5 μM and probe concentration of 0.25 μM if not otherwise optimised. |
| Improperly stored or expired reagents | <ul style="list-style-type: none"> • Check storage conditions and expire dates of the reagents. |
| Sample not configured properly | <ul style="list-style-type: none"> • Check the plate configuration. |
| Late increase in fluorescence signal | |
| Error in cycler setup | <ul style="list-style-type: none"> • Make sure that the instrument settings are correct for the experiment. |
| Insufficient starting template | <ul style="list-style-type: none"> • Check the calculation of the template stock concentration; increase the template amount if possible. |
| Improperly stored or expired reagents | <ul style="list-style-type: none"> • Check storage conditions and expiration dates of the reagents. |
| qPCR primer and/or probe design or concentration not optimal | <ul style="list-style-type: none"> • Check primer and probe design. |
| Annealing temperature too high | <ul style="list-style-type: none"> • Use gradient to optimize annealing temperature. • Decrease annealing temperature in 2°C decrements if a gradient feature is not available. |
| Insufficient extension time for the amplicon size | <ul style="list-style-type: none"> • Increase extension time. |
| Primer or probe concentration too low | <ul style="list-style-type: none"> • Increase primer concentration (to maximum of 1 μM each). 0.25 μM probe concentration is usually sufficient. |
| Insufficient activation of the hot start <i>Tbr</i> DNA polymerase | <ul style="list-style-type: none"> • Make sure 95°C 15 min was used for the initial denaturation step. • Make sure cycler block temperature is accurate. |
| PCR protocol not optimal | <ul style="list-style-type: none"> • Make sure the recommended PCR protocol is used. If necessary, optimize using the recommended protocol as a starting point. |

| Normal fluorescence signal, but low efficiency | |
|--|--|
| Pipetting error | <ul style="list-style-type: none"> • Check the assembly of the reactions. |
| Primer–dimers from previous run contaminating the reaction | <ul style="list-style-type: none"> • Perform UNG treatment before PCR cycling. |
| Primer and probe design not optimal | <ul style="list-style-type: none"> • Re-check primer and probe design. |
| Inhibitors from the sample affecting reaction | <ul style="list-style-type: none"> • Repurify DNA. |
| Low initial template concentration | <ul style="list-style-type: none"> • Increase template amount. |
| Non-linear correlation between C_q and log of template amount in the standard curve | |
| Template dilution inaccurate | <ul style="list-style-type: none"> • Remake dilution series and make sure the samples are well mixed. |
| Template amount too high | <ul style="list-style-type: none"> • Reduce template amount. • Increase reaction volume. |
| Template amount too low | <ul style="list-style-type: none"> • Increase the template amount. |
| Insufficient activation of the hot start <i>Tbr</i> DNA polymerase | <ul style="list-style-type: none"> • Make sure 95°C 15 min was used for the initial denaturation step. • Make sure cycler block temperature is accurate. |
| Insufficient denaturation of template | <ul style="list-style-type: none"> • Make sure 95°C 15 min was used for the initial denaturation step. • Make sure cycler block temperature is accurate. |
| Serious contamination | <ul style="list-style-type: none"> • Find contamination source and change contaminated components. |
| Low signal when using ROX normalization | |
| High ROX passive reference fluorescence intensity | <ul style="list-style-type: none"> • Use lower ROX concentration. See recommended concentrations in Table 2. |
| High signal when using ROX normalization | |
| Low ROX passive reference fluorescence intensity | <ul style="list-style-type: none"> • Use higher ROX concentration. See recommended concentrations in Table 2. |
| Yellow dye in the sample buffer decreases ROX intensity | <ul style="list-style-type: none"> • 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 | <ul style="list-style-type: none"> • Verify color calibration according to instrument instructions. |

Appendix I: cDNA synthesis

The cDNA synthesis step is very critical in qRT-PCR. The efficiency of reverse transcription varies and can be low in some cases. The expression level of the target RNA molecule and the efficiency of the RT reaction must therefore be considered when determining the appropriate amount of the starting template for subsequent PCR steps. The volume of cDNA template should not exceed 10 % of the qPCR reaction volume, as elevated volumes of cDNA reaction components 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 quantitation 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 and probe(s) 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 or probe(s) 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 quantitation ($\Delta\Delta C_q$ method), see Section 6.2.

The amplification efficiency of a reference gene should be the same as the amplification efficiency of the target gene, i.e. the slopes of their standard curves are identical. For efficiency calculation using the slope, see Section 6.1 (Absolute quantification).

Appendix II: general molecular biology data

Table 7. Spectrophotometric conversions for nucleic acid templates.

| 1 A_{260} unit* | Concentration ($\mu\text{g/ml}$) |
|---------------------|------------------------------------|
| Double-stranded DNA | 50 |
| Single-stranded DNA | 33 |
| Single-stranded RNA | 40 |

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

Table 8. Molar conversions for nucleic acid templates.

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

* For single-copy genes.

Product use limitation

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**Thermo Scientific DyNAmo Probe qPCR Kit
Technical Manual**



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v3_10.2011

Technical support:
US: techservice.genomics@thermofisher.com

Europe, Asia, Rest of World:
techservice.emea.genomics@thermofisher.com

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