Thermo Scientific
DyNAmo SYBR Green 2-Step qRT-PCR Kit

Technical Manual

F-430S   20 cDNA synthesis reactions (20 µl each)
          100 qPCR reactions (20 µl each)
F-430L   100 cDNA synthesis reactions (20 µl each)
          500 qPCR reactions (20 µl each)

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1. Description

Quantitative PCR (qPCR) is a useful technique for the investigation of gene expression, viral load, pathogen detection, and numerous other applications. When analyzing gene expression or viral load, the RNA of interest first needs to be reverse transcribed into cDNA. The following qPCR can either be performed separately (two-step qRT-PCR) or in the same tube with reverse transcription (one-step qRT-PCR).

Thermo Scientific DyNAmo SYBR Green 2-Step qRT-PCR Kit is designed for two-step qRT-PCR of RNA samples from various sources. The kit includes all the necessary reagents for cDNA synthesis and following SYBR Green qPCR. Either total RNA, messenger RNA, viral RNA or *in vitro* transcribed RNA can be used as a template for reverse transcription. The kit includes both random primers and oligo(dT) primers. The user can choose either of these or alternatively use gene specific primers.

The reverse transcriptase in the DyNAmo™ SYBR® Green 2-Step qRT-PCR Kit is M-MuLV RNase H+, which provides higher sensitivity to qPCR than RNase H- reverse transcriptases. The RNase H activity in the RT enzyme facilitates annealing of PCR primers to the cDNA by degrading the RNA template before the PCR step.

The performance of the qPCR step is based on a hot start version of a modified *Thermus brockianus (Tbr)* DNA polymerase and SYBR Green I fluorescent dye. A nonspecific DNA binding domain has been fused to the *Tbr* DNA polymerase. The domain lends physical stability to the polymerase-DNA complex. The initial denaturation step in the PCR protocol activates the modified hot-start *Tbr* polymerase. SYBR Green I is specific for double-stranded DNA and fluoresces when bound to the amplified double-stranded PCR product, thereby permitting the direct quantification of amplified DNA without labeled probes.

The reaction chemistry of DyNAmo SYBR Green 2-Step qRT-PCR Kit is applicable to most block-based real-time PCR 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 2-Step qRT-PCR Kit (F-440).

2. Kit components

cDNA synthesis	F-430S	F-430L
M-MuLV RNase H$^+$ reverse transcriptase (includes RNase inhibitor)	1 x 40 µl (sufficient for 20 RT reactions of 20 µl)	1 x 200 µl (sufficient for 100 RT reactions of 20 µl)
2x RT buffer (includes dNTP mix and 10 mM MgCl$_2$*)	1 x 200 µl	1 x 1 ml
Random hexamers (300 ng/µl)	1 x 20 µl	1 x 100 µl
Oligo(dT)$_{15}$ primer (100 ng/µl)	1 x 20 µl	1 x 100 µl
qPCR	**F-430S**	**F-430L**
2x master mix (contains modified hot-start *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

*   Provides 5 mM MgCl$_2$ in 1x reaction concentration
** Provides 2.5 mM MgCl$_2$ in 1x reaction concentration

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

3. Shipping and storage

The DyNAmo SYBR Green 2-Step qRT-PCR Kit is shipped on gel ice. Upon arrival, store all kit components at -20 °C. All the kit components can be refrozen and stored at -20 °C without affecting the performance of the kit.

4. cDNA synthesis

4.1 Notes about reaction components

Table 1. General recommendations.

Categories	Comments
Reaction volume	20 µl
Template amount	Max 1 µg of RNA.
Priming options	Random hexamers, oligo(dT) or gene-specific primers.

RNA template

Total RNA, mRNA, viral RNA or *in vitro* transcribed RNA can be used as a template. Successful cDNA synthesis is dependent on the integrity and purity of the template RNA. RNA preparation should be free of any DNA or RNase contamination. The purity of RNA can be determined by measuring the ratio of A_{260}/A_{280}. The optimal ratio is 1.8–2.0.

RNA isolation should be performed under RNase-free conditions. Furthermore, any contamination with RNases from other potential sources like glassware, plasticware and reagent solutions has to be avoided. This can be done by wearing gloves and using sterile tubes and pipet tips. Water used for the reactions should also be RNase free, but not DEPC treated as traces of DEPC can inhibit PCR.

DNA contamination can be removed from the RNA sample by treating the sample with RNase free DNase I. This should be done especially if primers for the qPCR step cannot be designed in exon-exon boundaries or in separate exons. See information about qPCR primer design in Section 5.1.

The maximum amount of template RNA for DyNAmo SYBR Green 2-Step qRT-PCR Kit is 1 µg. This amount includes all RNA present in the sample, for example carrier RNA and other possible RNAs in addition to the target RNA.

M-MuLV RNase H+ reverse transcriptase

M-MuLV RNase H+ RT provides good sensitivity in qRT-PCR applications, where amplicons are usually around 100 bp in length. Also, with M-MuLV RNase H+ there is no need to perform separate RNase H treatment after cDNA synthesis, as the RNase H activity in the enzyme degrades RNA in the RNA-cDNA hybrid.

RNase inhibitor

The RNase inhibitor included in the mix with the reverse transcriptase inhibits contaminating RNases present in the RNA sample. It does not affect the RNase H activity in the M-MuLV reverse transcriptase.

RT primers

Specific primers, random hexamers or oligo(dT) primers can be used for the RT step. Using specific primers can help to 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.

A good starting point is to use random hexamers for cDNA synthesis. Random hexamers transcribe all RNA (mRNA, rRNA, tRNA and *in vitro* transcribed RNA) producing cDNA that covers the whole transcript. The recommended amount of random hexamers per 20 µl RT reaction is 300 ng (can be optimized between 200–400 ng if necessary).

Oligo(dT) primers can be used to transcribe poly(A)+ RNAs. These include eukaryotic

mRNAs and retroviruses with poly(A)+ tails. Several different mRNAs are transcribed allowing subsequent qPCR detection of different targets from the same cDNA synthesis reaction. The recommended amount of oligo(dT) primers per 20 µl RT reaction is 100 ng (can be increased up to 1 µg if necessary). If the amplicon is located at the 5′ end of the transcript, using random hexamers is recommended.

Gene-specific primers are used to transcribe only the particular RNA of interest, in contrast to oligo(dT)/random primers that transcribe all mRNAs/RNAs in the sample. The recommended amount of specific primer per 20 µl RT reaction is 10 pmol (can be optimized between 5–20 pmol if necessary).

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.

RT efficiency

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 starting template for subsequent PCR steps. The volume of cDNA template should not exceed 10 % of the qPCR reaction volume, as elevated volumes of 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.

Standards

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 nucleic acid. (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 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.

Using RNA molecules as standards for RNA quantification is recommended. The use of RNA standards takes the variable efficiency of the reverse transcription into account. RNA standards can be generated for example by cloning the cDNA of interest to a vector containing RNA polymerase promoter, e.g. T7 or Sp6. From the vector the insert can be *in vitro* transcribed to obtain the final RNA standard with identical sequence to the

target amplicon. The vector must then be degraded with RNase-free DNase, and the concentration of the RNA standard determined spectrophotometrically. Alternatively a defined RNA preparation, e.g. from a cell line or a virus, with known concentration can be used as an RNA standard.

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.

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. If this is not the case, the results have to be corrected for the efficiency.

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. (2000) *Journal of Molecular Endocrinology* 25, 169–193
Bustin S.A. (2002) *Journal of Molecular Endocrinology* 29, 23-39.

4.2 Reaction setup

- Perform the reaction setup in an area separate from nucleic acid preparation and PCR product analysis.
- All plasticware should be RNase-free.
- Use gloves to prevent RNase contamination.
- Pipette with sterile filter tips.
- Minimize pipetting errors by using calibrated pipettes and by preparing premixes to avoid pipetting very small volumes.
- Pipet all components on ice.
- Reaction tubes should be centrifuged before starting the incubations to force the solution to the bottom of the tubes and to remove any bubbles.

Protocol

1. Program the cycler as outlined in Table 3.
2. Thaw template RNA, 2x RT buffer and primers. Mix the individual solutions to ensure homogeneity.
3. Prepare a cDNA synthesis premix by mixing 2x RT buffer, primers, RNase free H_2O and reverse transcriptase (see Table 2). Mix thoroughly to ensure homogeneity. Dispense appropriate volumes into reaction tubes.
4. Add template RNA to the reaction tubes.
5. Place the tubes in the thermal cycler and start the program.

Table 2. Reaction setup for cDNA synthesis.

Components	Stock	20 µl reaction	Comments
RT buffer	2x	10 µl	RT buffer includes dNTPs and $MgCl_2$
Random hexamer primer set	300 ng/µl	1 µl	Alternatively oligo(dT) primer or a specific primer can be used. See Section 4.1 ('RT primers').
Template RNA		X µl	Max 1 µg.
M-MuLV RNase H+ reverse transcriptase		2 µl	Includes RNase inhibitor.
RNase free H_2O		add to 20 µl	

Table 3. Cycling protocol for reverse transcription.

Step	Purpose	Temperature	Time	Comments
1	Primer extension	25°C	10 min	This step is not necessary if gene specific primers are used.
2	cDNA synthesis	37°C	30 min	Most targets can be synthesized at 37°C. The temperature can be varied between 37–48°C if necessary. Incubation time can be extended up to 60 min if needed for long or rare transcripts.
3	Reaction termination	85°C	5 min	Inactivation of M-MuLV prevents it from inhibiting qPCR reaction.
4	Cooling the sample	4°C	Hold	Optional

4.3 cDNA synthesis steps

Predenaturation (optional)
A separate RNA denaturation step is generally not required, but it can be performed before cDNA synthesis if the template RNA has a high degree of secondary structure. The denaturation step, at 65°C for 5 min, should be performed before adding 2x RT buffer and reverse transcriptase to the reaction mix.

Primer extension
The incubation at 25°C for 10 min extends random primers or oligo(dT) primers before the actual cDNA synthesis. Without the incubation at 25°C the primers may dissociate from the template when the temperature is raised to the cDNA synthesis temperature. This preliminary extension step is not necessary for gene-specific primers.

cDNA synthesis
Incubation at 37°C will work for most templates, but it can be optimized between 37°C and 48°C if necessary. Raising the temperature can be helpful if the template has strong secondary structures. Higher temperature can also improve specificity if gene-specific primers are used. Incubation time of 30 min is sufficient in most cases. If the target is located near the 5' end of a long transcript and oligo(dT) priming is used, or the target is rare, cDNA synthesis time can be extended up to 60 min.

Reaction termination
The termination step at 85°C inactivates the M-MuLV reverse transcriptase, thus preventing it from inhibiting the qPCR reaction.

5. qPCR

5.1 Notes about reaction components

Table 4. General recommendations for qPCR.

Categories	Comments
Consumables	Follow the recommendations of the PCR instrument manufacturer.
Reaction volume	20–50 µl
Amplicon size	50–250 bp
Template amount	The volume of cDNA template should not exceed 10 % of the qPCR reaction volume.
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 a modified 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.

cDNA template

If the cDNA synthesis reaction will not be used for qPCR immediately, it can be stored at -20°C. Also, if only part of the reaction volume is needed for qPCR, store the remainder at -20°C.

The volume of cDNA template should not exceed 10 % of the qPCR reaction volume, as elevated volumes of template may reduce the efficiency of the PCR amplification. Excess salt and random primers in the cDNA synthesis reaction can inhibit the DNA polymerase. A dilution series of the template can be made to optimize the amount of the starting material used.

DNA polymerase

The 2x qPCR master mix in the DyNAmo SYBR Green 2-Step qRT-PCR Kit includes a hot start version of a modified *Thermus brockianus* DNA polymerase. The modified polymerase incorporates a nonspecific DNA binding domain that lends physical stability to the polymerase-DNA complex. The modified *Tbr* polymerase is chemically engineered to be inactive at room temperature. The inactivation prevents the extension of non-specifically bound primers during reaction setup and therefore increases PCR specificity. The reaction setup can be performed at room temperature. The initial denaturation step in the PCR protocol reactivates the polymerase (hot start).

Primers for qPCR step

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

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.

ROX™ passive reference dye

For most real-time instruments ROX passive reference dye is not required, but on some instruments it is used to normalize for non-PCR related fluorescence signal variation. Passive reference dye does not take part in the PCR reaction and its fluorescence remains constant during the PCR reaction. The amount of the ROX passive reference dye needed can vary depending on the type of the 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 5 for instrument-specific recommendations). Note that the use of ROX passive reference dye may not be possible with some fluorescent dyes.

Table 5. 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)

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 before PCR. UNG (uracil-N-glycosylase) digests dU-containing DNA, and the digested DNA cannot act as a template in qPCR (Longo M.C. *et al.* (1990) *Gene* 93: 125–28). UNG is inactivated during the first denaturation step in PCR. The UNG treatment step (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 containing reaction products should not be opened or analyzed by gel electrophoresis in the same laboratory area that is used to set up reactions.

MgCl$_2$ optimization

Generally, it is not necessary to optimize the MgCl$_2$ concentration with the DyNAmo SYBR Green 2-Step qRT-PCR Kit. For most qPCR reactions, we recommend a final concentration of 2.5 mM MgCl$_2$, as provided in the master mix. However, in some rare cases improved results may be obtained with higher MgCl$_2$ concentrations. Excessive MgCl$_2$ concentrations can lead to amplification of nonspecific products and primer-dimers, however. Usually no more than 5 mM MgCl$_2$ is required by any amplicon.

5.2 Reaction setup

- Perform the reaction setup in an area separate from nucleic acid preparation and PCR product analysis.
- As the hot-start DNA polymerase is inactive during PCR setup, it is not necessary to do the setup on ice.
- Pipette with sterile filter tips.
- Minimize the exposure of the qPCR master mix to light.
- Minimize pipetting errors by using calibrated pipettes and by preparing premixes to avoid pipetting very small volumes.
- Use optically clear caps or sealers to achieve maximum signal.
- Use a cap sealing tool or firm finger pressure to close caps properly, or use a film sealer.
- Avoid touching the optical surface of the cap or sealing film without gloves, as fingerprints may interfere with fluorescence measurements.
- 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$_2$O.

5.2.1 General protocol for all instruments

If you are using an Applied Biosystems real-time PCR instrument, see Section 5.2.2.

Reaction setup

1. Program the cycler as outlined in Table 7.
2. Thaw the template cDNA, 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.
3. Prepare a PCR premix by mixing the master mix, primers, (ROX if used,) and H_2O see Table 6). Mix the PCR premix thoroughly to ensure homogeneity. Dispense appropriate volumes into strip tubes or plate wells.
4. Add template cDNA to the strip tubes or plate wells containing the PCR premix. 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 6. 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. Avoid air bubble formation.
Primer mix (in H_2O)	X μl	X μl	0.5 μM fwd 0.5 μM rev	Titrate from 0.3 to 1 μM if necessary.
50x ROX reference dye	(1 μl)	(0.4 μl)	1x	Optional. See Sections 5.1 and 5.2.2.
Template cDNA	X μl	X μl		Do not exceed 10 % of the final reaction volume. A dilution series of the cDNA synthesis reaction can be made to optimize the amount.
H_2O	add to 50 μl	add to 20 μl		

For different volumes, adjust all components proportionally.

Cycling protocol

Table 7. Cycling protocol.

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 DNA polymerase and to denature the template cDNA.
2	Denaturation	94°C	10 s	
3*	Annealing	X°C	20–30 s	5°C below lower primer Tm, use gradient feature to optimize.
4	Extension	72°C	30 s	Longer extension time may be necessary for amplicons > 500 bp. For two-step PCR, see Section 'Extension' on page 15.
5	Data acquisition			Fluorescence data collection
6	Number of cycles	35–45 cycles, steps 2–5		
10	Final extension			Optional, see page 15.
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 the instrument manufacturer's manual for detailed information.
12	Reannealing			Optional, see page 16.

* Use the Tm calculator at www.thermoscientific.com/pcrwebtools to determine Tm of the primers. Use 50 mM KCl and 0.5 µM primer concentration when calculating Tm (or the primer concentration in your reaction if optimized to other than 0.5 µM). Due to the characteristics of the modified DNA polymerase, it is often possible to use higher annealing temperatures than with other enzymes.

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 minutes is needed to ensure complete reactivation of the hot-start DNA polymerase and denaturation of the template.

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

Annealing

For most amplicons, annealing for 20–30 s 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 0.5 µM primer) calculated by the nearest-neighbor method as described by Breslauer *et al.* (1986) *Proc. Nat Acad. Sci.* 83: 3746–50. Instructions for Tm calculation and a link to a calculator using a modified nearest-neighbor method can be found on the Thermo Scientific website (www.thermoscientific. com/pcrwebtools). Different software may give different Tm 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.

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. Generally a 30 s extension time gives good results.

Depending on the amplicon a combined annealing/extension step can also be used (two-step PCR). Usually 1 min at 60°C works well for a combined annealing/extension step.

Data acquisition

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

Melting curve

A melting curve is used to check the specificity of an amplified product. When the temperature is gradually increased, a sharp decrease in SYBR Green fluorescence is observed as the product undergoes denaturation. Specific products can be distinguished from the nonspecific products by the difference in their melting temperatures. The recommended temperature ramp time is stated in Table 7. 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.

5.2.2 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.

Pipet 40 µl to each Master Mix tube

50 x ROX   Master Mix tubes

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.

Pipet 12 µl to each Master Mix tube

50 x ROX   Master Mix tubes
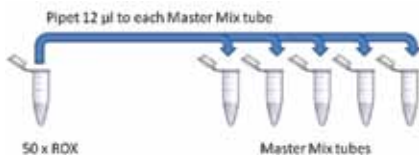
Reaction setup for all ABI models

1. Program the cycler as outlined in Table 9.
2. Thaw the template DNA, primers and 2x master mix (to which ROX passive reference dye has been added). Mix the individual solutions to ensure homogeneity. This is especially important for the master mix.
3. Prepare a PCR premix by mixing 2x master mix, primers, and H_2O. Mix the PCR premix thoroughly to ensure homogeneity. Dispense appropriate volumes into strip tubes or plate wells. Use the reverse pipetting technique to avoid bubbles.
4. Add template DNA (< 200 ng per 20 µl reaction) to the strip tubes or plate wells containing the PCR premix. For two-step qRT-PCR, the volume of the cDNA added (from the RT reaction) should not exceed 10 % of the final PCR volume.
5. Seal the strips or plate with an appropriate sealer, place them in the thermal cycler and start the cycling program.

Table 8. 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_2O)	X µl	X µl	0.5 µM fwd 0.5 µM rev	Titrate from 0.3 to 1 µM if necessary.
Template DNA (in H_2O)	X µl	X µl		Do not exceed 10 ng/µl in the final reaction.
H_2O	add to 50 µl	add to 20 µl		

Cycling protocol for all ABI models

Table 9. Cycling protocol for Applied Biosystems real-time PCR instruments.

Step	Temp.	Time	Cycles
Initial denaturation	95°C	15 min	1
Denaturation	95°C	10 s	40 cycles
Annealing/extension	60°C	60 s	
Dissociation curve as instructed by the instrument manufacturer			

Note: Make sure primers are designed suitably for a 2-step protocol. For a 3-step protocol, and for additional information about cycling steps, please refer to Section 5.2.1.

6. Analysis

6.1 Melting curve

Melting curve analysis is typically included in the analysis software of real-time fluorescence detection instruments. The melting point of the product depends mainly on base composition and length. When the decrease in SYBR Green fluorescence during the temperature increase is plotted as a negative first derivative, the temperature of the peak is defined as the Tm, or the melting temperature of the product.

If primer-dimers or other nonspecific products are observed, the efficiency of the PCR should be checked. Varying efficiency leads to incorrect quantification.

6.2 Absolute quantification

For RNA quantification, the use of standards is recommended (see 'Standards' in Section 4.1). Absolute quantification is performed by plotting samples of unknown concentration on a standard curve generated from a dilution series of template RNA of known concentration. Typically, the standard curve is a plot of the quantification cycle (Cq) against the logarithm of the amount of RNA. A linear regression analysis of the standard plot is used to calculate the amount of RNA in unknown samples. The slope of the equation is related to the efficiency of the PCR reaction. The PCR efficiency should be the same for standards and samples for quantification to be accurate. The PCR efficiency of the samples can be determined by doing a dilution series of these samples.

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

PCR efficiency = $((10^{\frac{-1}{slope}}) - 1) \times 100$ %
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 Section 4.1).

If the amplification efficiency of a reference gene is the same as that of the target gene, the comparative ΔΔCq method can be used for relative quantification. Both the sample

and the calibrator data are first normalized against variation in sample quality and quantity. Normalized (ΔCq) values are calculated by the following equations:

ΔCq(sample) = Cq(target) – Cq(reference)
ΔCq(calibrator) = Cq(target) – Cq(reference)

The ΔΔCq value is then determined using the following formula:
ΔΔCq = ΔCq(sample) –ΔCq(calibrator)

The expression of the target gene normalized to the reference gene and relative to the calibrator $= 2^{-\Delta\Delta Cq}$

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

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. • Check the concentrations and storage conditions of the reagents.
RNA degraded or poor quality	• Check the concentration, integrity, purity and storage conditions of the RNA template. Make new RNA dilutions from the stock if necessary.
Incorrect temperature in cDNA synthesis reactions	• The recommended temperature in cDNA synthesis step is 37°C. It can be optimized between 37–48°C if necessary.
Missing essential step in the cycler protocols	• Check the cycler protocols for cDNA synthesis and qPCR steps.
Reverse transcriptase not functional	• Make sure that the M-MuLV RNase H⁺ enzyme is not heat-inactivated. It should not be used in temperatures higher than 48°C.
RT-PCR product too long	• The lenght of the amplicon should be between 50 and 250 bp. The optimal lenght is 100–150 bp.
qPCR primer design or concentration not optimal	• Check primer design. See Section 5.1. • Use primer concentration of 0.3–1.0 µM.
Sample not configured properly	• Check the plate configuration.

Late increase in fluorescence signal	
Error in cycler setup	• Make sure that the instrument settings are correct for the experiment.
Missing components or pipetting error	• Check the assembly of the reactions. • Check the concentrations and storage conditions of the reagents.
RNA template amount too low	• Check the calculation of the template stock concentration; increase the amount of RNA template (max 1 μg).
RNA degraded or poor quality	• Check the concentration, integrity, purity and storage conditions of the RNA template. Make new RNA dilutions from the stock if necessary.
RNA template contains strong secondary structures	• Perform a predenaturation step on the template before cDNA synthesis. See Section 4.3.
Incorrect temperature in cDNA synthesis reaction	• The recommended temperature in cDNA synthesis step is 37°C. It can be optimized between 37–48°C if necessary.
Reverse transcriptase not functional	• Make sure that the M-MuLV RNase H$^+$ enzyme is not heat-inactivated. It should not be used in temperatures higher than 48°C.
RT-PCR product too long	• The length of the amplicon should be between 50 and 250 bp. The optimal length is 100–150 bp.
Insufficient activation of the hot start DNA polymerase	• Make sure 95°C 15 min was used for the initial reactivation/denaturation step. • Make sure cycler block temperature is accurate.
qPCR primer design not optimal	• Check primer design. See Section 5.1.
qPCR primer concentration too low	• Increase qPCR primer concentration (to a maximum of 1 μM each).
Annealing temperature too high in qPCR	• Use a gradient to optimize the annealing temperature. • Decrease the annealing temperature in 2°C decrements if no gradient feature is available.
PCR protocol not optimal	• Make sure you are using the recommended PCR protocol. If necessary, optimize using the recommended protocol as a starting point.
Increase in fluorescence signal in negative (no RT) control	
Contaminating genomic DNA in RNA preparation	• Treat the starting RNA template with DNase I before cDNA synthesis. • Redesign qPCR primers to prevent amplification of genomic DNA. See Section 5.1.
Primer-dimers or PCR products from a previous run contaminating the reaction	• Perform UNG treatment prior to PCR cycling.
Weak RT activity of the DNA polymerase	• Due to a weak RT activity of most DNA polymerases, short RNA target sequences might be reverse transcribed during qPCR step. • If contamination and amplification of genomic DNA can be excluded, you can ignore this no RT control.
Normal fluorescence signal, but melting curve analysis shows primer-dimers or nonspecific products only	
Missing components or pipetting error	• Check the assembly of the reactions. • Check the concentrations and storage conditions of the reagents.
Primer-dimers from a previous run contaminating the reaction	• Perform UNG treatment before PCR cycling.

Annealing temperature of qPCR primers too low	• Use gradient to optimize annealing temperature. • Increase annealing temperature in 2°C increments if no gradient feature is available.
qPCR primer design not optimal or very low cDNA template concentration	• Check primer design. See Section 5.1. • Increase cDNA template amount (up to 10 % of the qPCR reaction volume).

Normal fluorescence signal, melting curve analysis shows both primer-dimer or nonspecific product and specific product peaks	
Contaminating genomic DNA in RNA preparation	• Treat the starting RNA template with DNase I before cDNA synthesis. • Redesign qPCR primers to prevent amplification of genomic DNA. See Section 5.1.
Low initial cDNA template concentration	• Increase cDNA template amount (up to 10 % of the qPCR reaction volume).
qPCR primer design not optimal	• Check primer design. See Section 5.1.
Annealing temperature of qPCR primers too low	• Use gradient to optimize annealing temperature. • Increase annealing temperature in 2°C increments if a gradient feature is not available.
Primer-dimers or PCR products from previous run contaminating the reaction	• Perform 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 in qPCR too long	• Decrease extension time.

Non-linear correlation between Cq and log of template amount in the standard curve	
RNA template dilution inaccurate	• Remake dilution series and make sure the samples are well mixed.
RNA template amount too high	• Do not exceed 1 μg of starting RNA template.
RNA template amount too low	• Increase the amount of RNA template (max 1 μg).
RT-PCR product too long	• The lenght of the amplicon should be between 50 and 250 bp. The optimal lenght is 100–150 bp.
cDNA template volume too high	• Reduce template amount. The volume of the cDNA template should not exceed 10 % of the qPCR reaction volume. • Increase qPCR reaction volume.
cDNA template volume too low	• Increase template amount (up to 10 % of qPCR reaction volume).
Insufficient activation of the hot-start DNA polymerase	• Make sure 95°C 15 min was used for the initial reactivation/denaturation step in qPCR. • Make sure cycler block temperature is accurate.
Co-amplification of primer-dimers with the specific product	• Perform a second data acquisition at an elevated temperature to minimize the interference of primer-dimers.
qPCR primer design or concentration not optimal	• Check primer design. See Section 5.1. • Use primer concentration of 0.3–1.0 μM.

High initial fluorescence signal, gradually decreasing over the first 10–20 cycles	
Insufficient denaturation of cDNA template	• Make sure 95°C 15 min is used for the initial denaturation step in qPCR. • 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 5.
High signal when using ROX normalization	
Low ROX passive reference fluorescence intensity	• Use higher ROX concentration. See recommended concentrations in Table 5.
Abnormal appearance of amplification curves when ROX normalization is used	
Color calibration not accurate. Fluorescence intensity from one channel affects intensity in another channel.	• Verify color calibration according to instrument instructions.

Appendix I: general molecular biology data

Table 10. 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 11. 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}
Escherichia coli	4.7×10^{6} bp	3.2×10^{-4}	1.9×10^{8}
Human	3.2×10^{9} bp	4.7×10^{-7}	2.8×10^{5}

* For single-copy genes.

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