



MANUAL FOR
ONE STEP RT QPCR MASTERMIX

REFERENCE NUMBER: RT-0000-03



E U R O G E N T E C

EOT GROUP

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Shipping and storage conditions

All Eurogentec Real-Time qPCR kits including the One step RT qPCR MasterMix, are shipped on dry ice.

Upon receipt the One step RT qPCR MasterMix should be stored at -18 to -24 °C in a constant temperature freezer. When stored under these conditions the reagents are stable at least for six months. Twelve-month stability can be obtained by storage at -70 °C. The 2x reaction buffer and MgCl₂ can be stored at 4 °C up to one month, but the EuroScript Reverse Transcriptase requires storage at -20 °C or -70 °C to guarantee its performance. Repeated freeze-thaw cycles (more than 5 times) should be avoided.

Kit contents

The One step RT qPCR MasterMix (reference RT-QPRT-032X) contains sufficient RT qPCR reagents for up to 300 – 50 µl reactions using a hotstart enzyme, the HotGoldStar.

REAGENTS	VOLUME	DESCRIPTION
EuroScript Reverse Transcriptase (white cap)	75 µl	One tube of EuroScript RT, 3750 U at 50 U/µl and RNase Inhibitor, 1500 U at 20 U/µl
2x reaction buffer (red cap)	7.5 ml	Five tubes of reaction buffer containing dNTPs, HotGoldStar DNA polymerase, MgCl ₂ [5 mM final concentration], stabilizers and Passive Reference.
50 mM MgCl ₂ (plain cap)	1.5 ml	One tube of 50 mM MgCl ₂

Additional material required

The following items will be required in addition to the reagents supplied in the One step RT qPCR MasterMix:

- Real-Time thermocycler
- Nuclease free PCR-grade water
- Unlabelled primers
- Suitable RNase free plastic ware

Reagent handling and contamination control

Dispensing and mixing enzymes

Solutions containing enzymes should be mixed gently by inversion or gentle vortexing without creating bubbles.

Prevention of sample contamination

Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves. Change gloves whenever you suspect that they are contaminated.

Minimize the potential for carry-over of nucleic acid from one experiment to another by taking appropriate precautions. Use separate work areas and pipettes for pre- and post-amplification steps. Use aerosol-resistant pipette tips or positive displacement pipettes. Clean lab benches and equipment periodically with 10 % bleach solution.

Safety information

For more information about safety and handling of reagents please consult the Material Safety Data Sheets (MSDSs) of this kit and its components. These are available as PDFs on our website at www.eurogentec.com/code/en/msds.asp.

Background information

Numerous techniques have been developed to measure gene expression in tissues and cells. From these methods, RT qPCR is the most sensitive and versatile. The adaptation of PCR methodology to the investigation of RNA provides the researcher with a method featuring speed, efficiency, specificity and sensitivity. Since RNA cannot serve as a template for PCR, reverse transcription is combined with PCR to make complementary DNA (cDNA) suitable for PCR. This combination of both technologies is referred to as RT qPCR. The technique can be used to determine the presence or absence of a transcript and to quantify RNA expression levels.

One of the most powerful tools to have emerged to study the levels of gene expression is qPCR. A widely used form of this technology is Real-Time PCR using fluorescent probes. In Real-Time qPCR the signal generated is proportional to the amplicon concentration. A lot of different chemistries can be used such as Taqman® probes, Molecular Beacons or Scorpions® primers...

The Eurogentec One step RT qPCR MasterMix is a ready-to-use MasterMix, which allows both reverse transcription of the mRNA into cDNA, and the PCR amplification of this cDNA all in one single closed tube. The MasterMix format is ideal for high-throughput qPCR applications. This single tube one step format minimizes set-up time and avoids contamination. The use of the unique combination of EuroScript, RNase Inhibitor and hotstart HotGoldStar in the One step RT qPCR MasterMix provides a rapid and accurate method for Real-Time PCR.

The One step RT qPCR MasterMix has been designed for the Real-Time thermocyclers such as ABI Prism® SDS 5700, 7000, 7300, 7700, 7900 instruments and MasterCycler® ep Real Plex. But has also been validated on My iQ®, iCycler iQ®, iQ5, Mx4000®, Mx3000P®, Mx3005P®, DNA Engine Opticon® 1 and 2 systems, Mini Opticon®, Chromo 4, RotorGene 2000, 3000, 6000, Quantica® and LC480.

EuroScript Reverse transcriptase

EuroScript, a MmLV reverse transcriptase, gives reproducible high yields of full-length cDNA, which leads to a highly sensitive RT qPCR reaction over a wide range of RNA concentrations. This sensitivity is increased even more by the inclusion of RNase Inhibitor. In this way, any RNase activity is inhibited. In other kits containing RNase H(-) MmLV, the endogenous RNase activity is only inhibited while the RNA can still be degraded by other exogenous RNases. During this first denaturation step following the reverse transcriptase phase, the EuroScript Reverse Transcriptase is deactivated, avoiding any RT activity during the PCR reaction.

HotGoldStar DNA polymerase

The One step RT qPCR MasterMix contains HotGoldStar, a modified *Taq* polymerase, which allows hotstart PCR. It is delivered in its special buffer to perform under optimal conditions to produce a highly accurate, specific and sensitive PCR results.

The HotGoldStar is provided in an inactive form and remains inactivated during the reverse transcription reaction phase. This prevents misprimed RT PCR products as well as primer dimer formation during the reaction set-up and reverse transcription steps. Immediately following the reverse transcriptase step, the first denaturation step of the PCR phase activates the HotGoldStar polymerase.

2x reaction buffer

The 2x reaction buffer contains along with the HotGoldStar, dNTPs, MgCl₂, stabilizers and passive reference. The 2x reaction buffer has a special formulation, which allows both the reverse transcription reaction and the PCR reaction to take place in a very efficient way, in one single and the same tube.

The best results are obtained by using sequence specific primers during the reverse transcription and PCR. As the annealing of the primers can be influenced by concentration of MgCl₂, it might be necessary to optimize the concentration of MgCl₂. This can be done using the extra tube of 50 mM MgCl₂ delivered with this kit.

Passive Reference dye

The passive reference is used as an internal reference for normalization of the fluorescence signal, correction for well-to-well variations due to pipetting inaccuracies and fluorescence fluctuations. The use of a passive reference is necessary when using the ABI Prism® SDS 7900, 7700, 7300, 7000 ABI GeneAmp® 5700 SDS or MasterCycler®ep Real Plex. A passive reference can be used on the Mx4000®, Mx3000P®, Mx3005P® but is not necessary. On the My iQ®, iCycler iQ®, iQ5, DNA Engine Opticon® 1 and 2, Mini Opticon®, Chromo 4, RotorGene 2000, 3000 and 6000, Quantica® and LC480 a passive reference is not required, but the signal caused by the passive reference will be read in the channel where ROX is usually detected. The passive reference will not interfere with all other dyes detected in the other channels.

UNG

The use of Uracil-N-glycosylase (UNG) in a One step RT qPCR kit is NOT recommended, as the cDNA will be degraded upon reverse transcription.

Standard protocol

This protocol has been optimized for the ABI GeneAmp® SDS 5700 and ABI Prism® SDS 7000, 7300, 7700, 7900 and MasterCycler® ep Real Plex

However, it can also be used without modifications to the reaction temperatures and reaction times on the My iQ®, iCycler iQ®, iQ4, iQ5, Mx4000®, Mx3000P®, Mx3500P®, DNA Engine Opticon® 1 and 2 systems, Mini Opticon®, Chromo 4, RotorGene 2000, 3000 and 6000, Quantica® and LC480.

For the use of an ABI 7500, this kit is not convenient:

Please refer to our One step RT qPCR MasterMix Low Rox (RT-QPRT-032XLR) manual

When performing a One step RT qPCR assay, attention must be paid to the following issues

- The length of the amplicon should ideally be between 80 and 150 bp and not exceed 500 bp.
- Include an initial activation step of 10 minutes 95 °C between the RT and PCR reaction.
- Set up the reaction on ice to avoid premature cDNA synthesis at incorrect temperatures. This will cause mispriming of the oligos, which will lead to aspecific products. Set up reaction mixture in a different area from the one used for RNA preparation or RT PCR product analysis.
- Perform RNA isolation and reaction set-up in a RNase free environment.
- Use disposable pipette tips containing hydrophobic filters or positive displacement pipettes to prevent cross contamination.
- Start the initial concentration of Mg²⁺ at 5 mM as provided in the 2x MasterMix. However if a higher concentration of Mg²⁺ is required, use the stock of 50 mM of MgCl₂ for further optimization.

The following protocol has been optimized for 50 µl reactions; if other volumes are used, adjust the volumes of all components to keep the same concentrations, except for the primers and probe. For the primers and probe it might be necessary to keep the same absolute amount instead of the same concentration.

Do not use Uracil-N-glycosylase with these reagents. UNG will immediately degrade the cDNA produced upon reverse transcription.

Protocol

1. Thaw all required reagents completely and put them on ice except the EuroScript reverse transcriptase and RNase Inhibitor, which should be kept in the freezer until required for use. Mix well all reagents by inversion prior to pipetting.
2. Prepare a volume of MasterMix 10 % greater than required for the total number of reactions to be performed.
3. Prepare a reaction mix by combining the following components in order as detailed below:

COMPONENT	VOLUME (μ l)	FINAL CONCENTRATION
2x reaction buffer	25	1x
Forward primer	5	(starting with 300 nM)*
Reverse primer	5	(starting with 300 nM)*
Probe	5	(starting with 100 nM)*
EuroScript RT & RNase Inhibitor	0.25	0.25 units / μ l 0.1 Units / μ l
Template (added at step 5)	Variable	10 pg – 100 ng total RNA
RNase Free Water Adjust to 50 (including Template)	Variable	Volume is 50 μ l minus all other component volume.
Total MIX	50	

() Note that the primer and probe concentrations mentioned in this manual are recommended as starting concentrations. These concentrations will be correct for many assays, but additional optimization of the primer probe concentration may be required to obtain the best results with your primer-probe set.*

4. A negative control containing no RNA template should always be included. Optionally, a no RT-control should be set up in tubes / wells, which do not contain the EuroScript RT / RNase Inhibitor. This control is used to determine if there is any genomic DNA contamination in the RNA template.
5. Make sure that your reaction mix is thoroughly mixed (do not vortex), and aliquot the

Absolute or relative quantitation of target

Quantification of specific gene expression can be done either by absolute quantification or relative quantification. Absolute quantification determines the absolute copy number or amount of target. Relative quantification determines the ratio between the amount of target and a reference molecule.

Absolute quantification is determined using external standards. Using a dilution series of standards, a standard curve is derived by plotting Ct values against log of amount of standard. Amplification of the target and dilution series of standards is carried out in separate tubes. Comparison of the Ct of unknown amount of target with the standard curve permits determination of the initial amount of target used in amplification.

In relative quantification the ratio between the amount of target and a reference molecule within the same sample is calculated. Pivotal to this comparison is the assumption that the level of expression of the reference molecule, such as a housekeeping gene, must not vary under the different experimental conditions being studied. This level is used as a reference value for quantification. Different relative quantification procedures must be used depending upon whether the target and reference molecules are amplified with similar or different efficiencies. For further discussion go to the following website: www.gene-quantification.info or www.gene-quantification.com.

Experimental parameters to be considered

Negative controls:

1. A minus RT control is used for the detection of genomic DNA. If a product is seen in the minus RT control, it indicates that contaminating genomic DNA is present in the sample.
2. Another important control reaction is a minus template control. The minus template control includes all of the RT qPCR reagents except the RNA template. If a product is amplified, it indicates that one or more of the RT qPCR reagents are contaminated with RNA or DNA.

Use a MasterMix whenever possible: the use of a MasterMix will keep variability to minimum. Assembly of a MasterMix reduces pipetting inaccuracies and risks of contamination.

Troubleshooting guide

This troubleshooting guide takes you through the most common difficulties you can meet when performing a One step RT qPCR assay with probes. Our Customer Help Desk scientists are happy to help you if you might have any further questions (info@eurogentec.com / info.uk@eurogentec.com / info.us@eurogentec.com).

Growth curve – Ct / crossing point

No Ct value...

RT reaction temperature

We recommend to perform the reverse transcription reaction at 48 °C. However, the temperature can be adjusted (between 46 °C to 50 °C) to obtain better yields of cDNA.

Primers or probe are degraded

Check for degradation of primers and probes via denaturing PAGE.

Insufficient amount of starting template

Increase the amount of starting material, if possible.

Incorrect ratio between reverse transcriptase and PCR components

The volume of RT enzyme added should be 10 % maximum of the final PCR volume.

ΔT_m between forward and reverse primer is too high

If no amplification occurs, check ΔT_m between forward and reverse primers. If ΔT_m is more than 4 °C the PCR will not perform well or not at all.

UNG has been added to a One step kit

In the One step RT qPCR MasterMix, RNA is transcribed into cDNA. dUTPs are incorporated into the cDNA during this process at 48 °C. Because UNG optimum temperature is 52 °C it will immediately hydrolyze the dUMP containing cDNA.

Number of cycles is insufficient

Start with a minimum of 35 cycles, and then increase up to 45 cycles. More than 45 cycles will increase the background.

Detection during wrong PCR step

Make sure that detection occurs during annealing step (at 60 °C).

Primer and probe design suboptimal

Check via gel electrophoresis for presence of any PCR product.

If no specific products can be detected redo the design. To do so, use primer design software that checks for primer/probe T_m, complementarity, secondary structures, amplicon size, number of identical nucleotides, etc...

When studying expression profiles in eucaryotic targets: choose PCR primers that span at least one exon-exon junction in the target mRNA to prevent amplification of the target from contaminating genomic DNA.

The T_m of the probes should be 8-10 °C above the T_m of the primers. If the T_m of the probe is too low, the elongation will take place before the probe binds to the target and therefore the efficiency of the 5' nuclease assay will decrease. If a specific product can be seen redo the probe design as the probe does not hybridize in the present conditions.

If it is not possible to redo the design, try a 3-step protocol as described p.18, this could help to obtain better results.

For further information see our primer design guidelines p.26.

Late Ct value...

Efficiency is poor

This is usually caused by the length of the amplicon. For Real-Time PCR, the length of amplicons should be between 80 and 150 bp. With adjusted reaction times it is possible to amplify up to 500 bp.

Primer and probe design is not optimal or primers have been designed containing secondary structures.

If possible, we recommend to design a new primer according to the primer design guidelines (p.26). If not, try a 3-step protocol as described (p.18), this could enhance the PCR efficiency.

Poor quality of the starting template

Poor quality RNA can limit the efficiency of the reverse transcription reaction and reduce yields, giving late Ct values.

Check concentration, storage and purity conditions (see p.30). Prepare serial dilution series of the RNA template from the stock solution and repeat the reaction using new dilutions.

Use commercially available RNA extraction kits to obtain good quality RNA.

PCR annealing / extension time too short

Start with the recommended annealing / extension time (60 seconds) and increase with 10-second steps.

PCR annealing / extension temperature too high

Decrease annealing / extension temperature in steps of 2 °C.

PCR annealing / extension temperature too low

Increase annealing / extension temperature in steps of 2 °C.

RT reaction temperature

We recommend to perform the reverse transcription reaction at 48 °C. However, the temperature can be adjusted (between 46 °C to 50 °C) to obtain better yields of cDNA.

PCR product too long

Ideally a PCR product for Real-Time qPCR should be between 80 and 150 bp. However, with adjusted times it is possible to amplify products up to 500 bp.

Primers and probe design sub-optimal

Check via gel electrophoresis for presence of any PCR product.

If no specific product can be detected redo the design. To do so, use primer design software that checks for primers/probe T_m, complementarity, secondary structures, amplicon size, number of identical nucleotides, etc...

When studying expression profiles in eucaryotic targets: choose PCR primers that span at least one exon-exon junction in the target mRNA to prevent amplification of the target from contaminating genomic DNA.

The T_m of the probes should be 8-10 °C above the T_m of the primers

If it is not possible to redo the design, try a 3-step protocol as described p.18, this could help to obtain better results.

For further information see our primer design guidelines p.26.

Primer concentration or ratio sub-optimal

Optimize according to the table 1.

By doing this type of optimization it is also possible to compensate for differences up to 2°C in melt temperature of the primers.

Table 1. Primer optimization matrix

REVERSE	FORWARD		
	50 nM	300 nM	900 nM
50 nM	50/50	300/50	900/50
300 nM	50/300	300/300	900/300
900 nM	50/900	300/900	900/900

Primer-probe ratio sub-optimal

Select optimal primer concentration as described above and adjust probe concentration like in table 2.

Table 2. Primer-probe optimization matrix

	PROBE		
	50 nM	100 nM	250 nM
Opt. Primers	50 / opt.	100 / opt.	250 / opt.

Primers or probe are degraded

Check for degradation of primers and probe via denaturing PAGE.

Probe located at 5' end of amplicon

If the probe is located at the 5' end of the amplicon it will not be cleaved efficiently, and a weak signal will be generated.

Redesign and put the probe close to the 3' end of the amplicon.

The probe may be bleached if it has been left in the light for some time

Although the reaction is working the fluorophore is no longer reporting the result.

Dye-labelled oligonucleotides should be aliquoted and stored in the dark at -20 °C.

Freeze thawing should be avoided (no more than 5 freeze – thaw cycles).

Probe hydrolysis

When probes are dissolved in an acid solution, the fluorophores can be hydrolysed.

This will generate a low fluorescence signal and a high background (lower ΔR_n).

We recommend resuspending qPCR probes in TE buffer 0.01M pH 8.0 instead of water.

Insufficient amount of starting template

Increase the amount of starting material, if possible.

Typical starting concentrations are 10 pg – 50 pg of total RNA

DNA Template is degraded

Check DNA concentration on agarose gel for degradation. Check storage conditions if DNA is degraded and prepare new DNA.

Incorrect ratio between reverse transcriptase and PCR components

The volume of RT enzyme added should be 10 % maximum of the final PCR reaction volume.

MgCl₂ concentration suboptimal

Adjust concentration in 0.5 mM steps, starting from the concentration recommended on the Technical Data Sheet.

Pipetting errors

As RT qPCR is a highly sensitive tool, errors can also easily be amplified.

The use of MasterMixes can reduce this effect, as variability is kept to a minimum.

A standard curve should always be used to check for irregularities (for RNA quantitation or for verification of the efficiency for comparative quantitation).

To check for PCR efficiency and for pipetting errors refer to the PCR efficiency chapter p.29.

No linearity in the Ct values of a dilution series

Template amount is too high

Do not exceed maximum recommended amounts of RNA. We recommend to use less than 100 ng total RNA, final amount, per reaction.

Incorrect ratio between reverse transcriptase and PCR components

The volume of RT enzyme added should be 10 % maximum of the final PCR reaction volume.

Secondary structures in probes

For a 2x dilution series a ΔCt of 1 cycle should be seen between each dilution in the growth curve and for a 10x dilution series a ΔCt of 3.2 cycles should be seen. If there is a gap in these values, this can be caused by secondary structures in the probe. At the point where the gap occurs the target DNA amount is no longer in excess or balanced with the amount of probe. Therefore, a competition between intra-probe binding and target-probe binding starts, leading to less efficient detection. The only solution to this is redesigning the probe.

Growth curve - Rn / fluorescence

Low ΔRn ...

PCR annealing / extension time too short

Start with recommended annealing / extension time (60 seconds) and increase with 10-second steps.

PCR annealing / extension temperature too high

Decrease annealing/extension temperature in steps of 2 °C.

PCR annealing / extension temperature too low

Increase annealing/extension temperature in steps of 2 °C.

3-step protocol instead of 2-step protocol

If your PCR efficiency is still not good after varying annealing and / or extension times and temperatures, try a 3-step protocol as follows:

40 cycles	denaturation	15 s	95 °C
	annealing	30 s	60 °C
	extension	30 s	72 °C

Increase extension time with 10-second steps.

Primers and probe design sub-optimal

Check via gel electrophoresis for presence of any PCR product.

If no specific products can be detected redo the design. To do so, use primer design software that checks for primers/probe T_m, complementarity, secondary structures, amplicon size, number of identical nucleotides, etc...

When studying expression profiles in eucaryotic targets: choose PCR primers that span at least one exon-exon junction in the target mRNA to prevent amplification of the target from contaminating genomic DNA.

The T_m of the probes should be 8-10 °C above the T_m of the primers

If it is not possible to redo the design, try a 3-step protocol as described p.18, this could help to obtain better results.

For further information see the primer design guidelines p.26.

Primer concentration or ratio sub-optimal

Optimize according to the table 1.

By doing this type of optimization it is also possible to compensate differences up to 2 °C in melt temperature of the primers.

Refer to Table 1 p.15: Primer optimization matrix

Primer-probe ratio sub-optimal

Select optimal primer concentration as described above and adjust probe concentration like in table 2.

Refer to Table 2 p.16: Primer-probe optimization matrix

Probes hydrolysis

When probes are dissolved in an acid solution the fluorophores can be hydrolysed and will give a low fluorescence signal and a high background (lower ΔR_n).

Eurogentec recommends resuspending qPCR probes in TE buffer 0.01M pH 8.0 instead of water.

Probe located at 5' end of amplicon

If the probe is located at the 5' end of the amplicon it will not be cleaved or opened efficiently, and a weak signal will be generated.

Design a new probe closed to the 3' end of amplicon or on the opposite strand.

The probe may have bleached if it has been left in the light for some time

Although the reaction is working the fluorophore is no longer reporting the result. (lower signal at first... And late Ct values if the probes are really damaged).

Dye-labelled oligonucleotides should be aliquoted and stored in the dark at -20 °C. Freeze thawing should be avoided (no more than 5 freeze – thaw cycles).

Poor quality of the starting template

Check concentration, storage and purity conditions (see p.30). Prepare serial dilution series of the RNA template from the stock solution and repeat the reaction using new dilutions.

Use commercially available RNA extraction kits to obtain good quality RNA

If any inhibitory components are present in the RNA, the effect should decrease when increasing the dilution factor.

Insufficient amount of starting template

Increase the amount of starting material, if possible.

Typical starting concentrations are 10 pg – 50 pg of total RNA.

Incorrect ratio between reverse transcriptase and PCR components

The volume of RT enzyme added should be 10 % maximum of the final PCR reaction volume.

MgCl₂ concentration suboptimal

Adjust concentration in 0.5 mM steps, starting from the concentration recommended on the Technical Data Sheet.

Pipetting errors

As RT qPCR is a highly sensitive tool, errors will be quickly amplified. The use of MasterMixes can reduce this effect.

A standard curve should always be used to check for irregularities (for RNA quantitation or for verification of the efficiency for comparative quantitation).

Check for PCR efficiency and for pipetting errors.

Shallow growth curve

PCR annealing / extension time too short

Start with recommended annealing / extension time (60 seconds) and increase with 10-second steps.

3-step protocol instead of 2-step protocol

If your PCR efficiency is still not good after varying annealing and / or extension times and temperatures, try a 3-step protocol as follows:

40 cycles	denaturation	15 s	95 °C
	annealing	30 s	60 °C
	extension	30 s	72 °C

Increase extension time with 10-second steps.

PCR product too long

Ideally a PCR product for Real-Time qPCR should be between 80 and 150 bp. However, with adjusted times it is possible to amplify products up to 500 bp.

Primers and probe design sub-optimal

Check via gel electrophoresis for presence of any PCR product.

If no specific products can be detected redo the design. To do so, use primer design software that checks for primers/probe T_m, complementarity, secondary structures, amplicon size, number of identical nucleotides, etc...

When studying expression profiles in eucaryotic targets: choose PCR primers that span at least one exon-exon junction in the target mRNA to prevent amplification of the target from contaminating genomic DNA.

The T_m of the probes should be 8-10 °C above the T_m of the primers.

If it is not possible to redo the design, try a 3-step protocol as described p.18, this could help to obtain better results.

For further information see the primer design guidelines p.26.

Primer concentration or ratio sub-optimal

Optimize according to the table 1.

By doing this type of optimization it is also possible to compensate for differences up to 2 °C in melt temperature of the primers.

Refer to Table 1 p.15: Primer optimization matrix

Primer-probe ratio sub-optimal

Select optimal primer concentration as described above and adjust probe concentration like in table 2.

Refer to Table 2 p.16: Primer-probe optimization matrix

Probes hydrolysis

When probes are dissolved in an acid solution the fluorophores can be hydrolysed and will give a low fluorescence signal and a high background (lower ΔR_n).

We recommend resuspending qPCR probes in TE buffer 0.01M pH 8.0 instead of water.

Probe located at 5' end of amplicon

If the probe is located at the 5' end of the amplicon it will not be cleaved or opened efficiently, and a weak signal will be generated.

Redesign the probe to 3' end of amplicon.

The probe may be bleached if it has been left in the light for some time.

Although the reaction is working the fluorophore is no longer reporting the result. (lower signal at first... And late Ct values if the probes are really damaged).

Dye-labelled oligonucleotides should be aliquoted and stored in the dark at -20°C . Freeze thawing should be avoided (no more than 5 freeze – thaw cycles).

Poor quality of the starting template

Check concentration, storage and purity conditions (see p.30). Prepare serial dilution series of the RNA template from the stock solution and repeat the reaction using new dilutions.

Use commercially available RNA extraction kits to obtain good quality RNA.

If any inhibitory components are present in the RNA, this should decrease when increases the dilution factor.

High fluorescence in the negative control (no template control)

Contamination with DNA or PCR products from previous PCR of the MasterMix

Clean working practices should be used to avoid DNA template contamination.

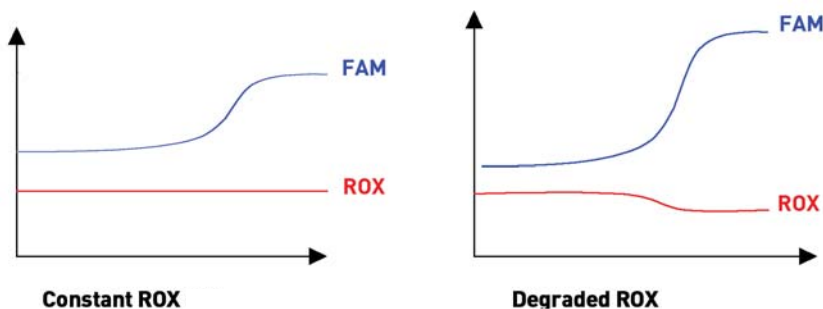
Probe has degraded because of heating

The signal will increase.

Run an agarose gel; if there is no product on gel, the probes are degraded.

ROX dye has degraded

On the multicomponent view the FAM signal will increase as ROX is degrading.



When detecting bacterial sequences only

If detecting bacterial sequences but not E. coli

Design of the primers has to exclude any part of the sequence that is shared between *E. coli* and the bacteria used (in other words, the primers have to be design only in the part, which is unique for the bacteria used).

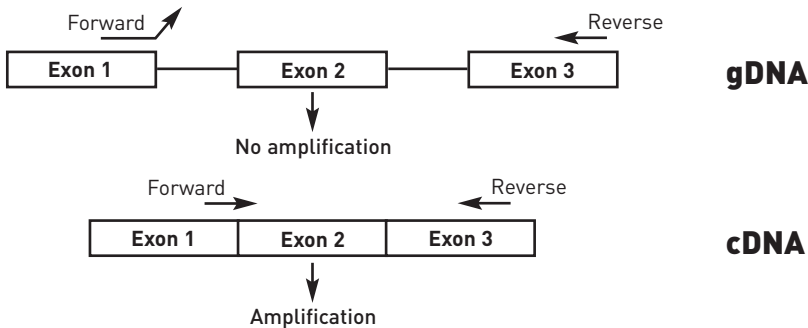
If detecting E. coli

In some recombinant *Taq* polymerases produced in *E. coli* some traces of bacterial DNA can be found. You will have to determine the minimum level and subtract it from the positive signal. Everything above this signal should be considered as positive.

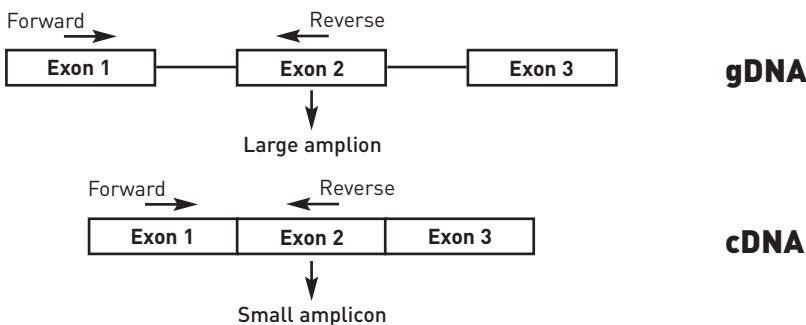
High fluorescence in no reverse transcriptase control

Contamination with genomic DNA

To avoid contamination with genomic DNA, treat the RNA with RNase free DNase and design primers, which are intron spanning to ensure amplification of only cDNA (see figure below and the primer design guidelines p.26).



It is also possible to design the forward and reverse primers to be in different exons. This will lead to a difference length of the amplicon, but not the exclusion of amplification of gDNA.



Standard curve

$R^2 < 0.9$

Pipetting errors

As RT qPCR is a highly sensitive tool, errors will be quickly amplified. The use of MasterMixes can reduce this effect.

A standard curve should always be used to check for irregularities (for RNA quantitation or verification of the efficiency for comparative quantitation).

To check PCR efficiency and pipetting errors refer to our PCR efficiency chapter p.29.

Imprecise dilutions

Prepare a high concentrated stock of your RNA preferably with a carrier like lambda DNA, tRNA or BSA, aliquote it to avoid freeze / thawing and keep them at $-20\text{ }^{\circ}\text{C}$ (preferably at $-80\text{ }^{\circ}\text{C}$).

For each Real-Time qPCR prepare a fresh dilution series out of the RNA template stock solution.

Poor quality of the starting template

Check concentration, storage and purity conditions (see p.30). Prepare dilution series of the RNA template from the stock solution and repeat the reaction using new dilutions.

Use commercially available RNA extraction kits to obtain good quality RNA.

If any inhibitory components are present in the RNA, the effect should decrease when the dilution factor increases.

Primers and probe design sub-optimal

Check via gel electrophoresis for presence of any PCR product.

If no specific products can be detected redo the design. To do so, use primer design software that checks for primers/probe T_m , complementarity, secondary structures, amplicon size, number of identical nucleotides, etc...

When studying expression profiles in eucaryotic targets: choose PCR primers that span at least one exon-exon junction in the target mRNA to prevent amplification of the target from contaminating genomic DNA.

The T_m of the probes should be $8\text{-}10\text{ }^{\circ}\text{C}$ above the T_m of the primers. If it is not possible to redo the design, try a 3-step protocol as described p.18, this could help to obtain better results.

For further information see our primer design guidelines p.26.

Secondary structures in the primers

Refer to "primer and probe-design suboptimal"

For further information see the primer design guidelines p.26.

Primer concentration or ratio suboptimal

Optimize according to the table 1 p.15.

By doing this type of optimization it is also possible to compensate for differences up to 2 °C in T_m of the primers.

Refer to Table 1 p.15: Primer optimization matrix

Probe is designed with secondary structures

For a 2x dilution series a Δ Ct of 1 cycle should be seen between each amplification plot on the growth curve and for a 10x dilution series a Δ Ct of 3.2 cycles should be seen. If there is a gap in these values, this can be caused by secondary structures in the probe. At the point where the gap occurs the target DNA amount is no longer in excess or balanced with the amount of probe. Therefore, a competition between intra-probe binding and target-probe binding starts leading to less efficient detection. The only solution to this is redesigning the probe.

Slope > -3.32 (< 100 % PCR efficiency)

Secondary structures in the primers, probe or amplicon

Refer to “primer design suboptimal”.

For further information follow the usual primer and probe guidelines p.26.

PCR product too long

Ideally a PCR product for Real-Time PCR should be between 80 and 150 bp. However, with adjusted times it is possible to amplify products up to 500 bp

Slope < -3.32 (> 100 % PCR efficiency)

Probe has degraded because of heating

The signal will increase.

Run an agarose gel; if there is no product on gel, the probes are degraded.

Non linear standard curve

Template amount is too high

Do not exceed maximum recommended amounts of RNA. We recommend using less than 100 ng total RNA final concentration per reaction.

Secondary structures in the primers, probe or amplicon

Refer to “primer design suboptimal”.

For further information follow the usual primer design guidelines p.26..

Probe are designed with secondary structures

For a 2x dilution series a ΔC_t of 1 cycle should be seen between each amplification plot on the growth curve and for a 10x dilution series a ΔC_t of 3.2 cycles should be seen. If there is a gap in these values, this can be caused by secondary structures in the probe. At the point where the gap occurs the target DNA amount is no longer in excess or balanced with the amount of probe. Therefore, a competition between intra-probe binding and target-probe binding starts leading to less efficient detection. The only solution to this is redesigning the probe.

Disbalanced between primer and template concentration

If primers are in strong excess of amplicon this will generate primer dimers and will disturb the linear relationship.

GC rich amplicon

Add up to 5 % DMSO to decrease melting temperature of amplicon

Inhibitors in DNA

Inhibitors will be diluted out in lower concentration range, therefore linearity will become better towards the more diluted RNA range.

Primer design guidelines

Well-designed primers and probes are a prerequisite for successful RT qPCR. By using well-designed primers and probes PCR efficiencies of 100 % can be obtained when using Eurogentec RT qPCR or qPCR kits.

If the following primer design guidelines are taken into account you will achieve high PCR efficiencies, specific PCR products, non co-amplification of gDNA and therefore the most sensitive results.

We recommend in general using a design software (for example primer 3 or Oligo® 6.0) to check for all following criteria.

In probe assays primer dimers and non-specific products will not be detected, however, they will influence the PCR dynamics and efficiency. Therefore also in probe assays they should be avoided as much as possible. For probe assays the amplicons should be kept as short as possible, with the 5' end of the probe as close as possible to the 3' end of the forward primer in case the probe is on the same strand, and as close as possible to the 3' end of the reverse primer in case the probe is on the opposite strand. In this way the 5' nuclease reaction will be optimal.

Experience has learned that it is easier to first design the probe and then the primers than the other way around.

By selecting quenchers that fit the fluorophores used with the Double-Dye oligos you will be able to obtain high signal. Eclipse® DarkQuencher and Black Hole Quencher® give better signal-to-noise ratios than TAMRA. We, in general, recommend taking fluorophores that can be coupled efficiently to the oligo during the synthesis. Examples of fluorophores which can be coupled efficiently are FAM, HEX, TET, Yakima Yellow®, Texas Red®, Cy®3 and Cy®5. ROX is an example of label, which is hard to couple efficiently.

Probes

- Length
 - 18-30 bases
 - Optimal: 20 bases
 - Lengths over 30 bases are possible, but the position of the quencher is recommended to be internally between 18-25 bases from the 5' end
- GC content
 - 30-80 %

- T_m
 - T_m of the probe must be 8-10 °C (8 °C for genotyping, 10 °C for expression profiling) higher than the T_m of the primers
- Select the strand that gives the probe more Cs than Gs
- Place probe as close as possible to primers without overlapping them
- Avoid mismatches between probe and target
- Avoid complementarity with either of the primers
- Avoid runs of identical nucleotides, especially of 4 or more Gs
- Avoid 5' end G (quenches the fluorophore)
- For multiplex assays:
 - position the polymorphism in the center of the probe
 - adjust the probe length so that both probes have the same T_m

Primer

- Length:
 - 18 - 30 bp
- GC content: 30-80 % (ideally 40-60 %)
- T_m
 - 63-67 °C (ideally 64 °C) ($T_m = 2(\text{number A+T}) + 4(\text{number G+C})$), so that Annealing is 58-62 °C (ideally 59 °C)
 - ΔT_m forward primer and reverse primer ± 4 °C
- Avoid mismatches between primers and target, especially towards the 3' end of the primer
- Avoid runs of identical nucleotides, especially of 3 or more Gs or Cs at the 3' end
- Avoid 3' end T (allows mismatching)
- Avoid complementarity within the primers to avoid hairpins (check with Oligo®)
- Avoid complementarity between the primers to avoid primer dimers, especially at 2 or more bases at the 3' ends of the primers (check with Oligo®)
- Design intron spanning or intron flanking primers to prevent or identify amplification of contaminating genomic DNA (see figure p.22) for intron spanning primers the first half of the oligo must hybridize to the 3' end of one exon and to the 5' end of the other exon. In this way only cDNA will be amplified and gDNA not. For intron flanking primers the forward primer must hybridize to one exon and the reverse primer to the other exon. Amplicons from cDNA, without introns, will be smaller than the amplicons from gDNA, which will contain the introns. The difference in size of these amplicons can be determined via meltcurve analysis. If genes of only one exon are studied contamination with gDNA can only be avoided by DNase treatment of the RNA with RNase free DNase.
- Check if primers are unique and specific (check with BLAST: www.ncbi.nlm.nih.gov/BLAST/)

Amplicon

- Length
 - 80-120 bp optimal (up to 500-1000 bp possible with adjusted reaction times)
 - shorter amplicons will give higher PCR efficiencies and more efficient 5' nuclease reactions
- GC content: - 30-80 % (ideally 40-60 %)
- Avoid secondary structures in the amplicon (check with Mfold: www.bioinfo.rpi.edu/applications/mfold/).

How to set up a good assay?

PCR efficiency

In order to get accurate quantitation and reproducible results, Real-Time qPCR reactions should have an efficiency close to 100 %.

The PCR efficiency is influenced by the follows:

- Length of amplicon
- GC content of amplicons
- Secondary structures of primers and probe and / or amplicon
- Concentration of reaction components

During the development of the One step RT qPCR MasterMix, Eurogentec has designed a kit that works at 100 % efficiency, when using the correct primers and probe. Good primers and probe can be found when following the design guidelines as mentioned on p.26. These primers can be checked easily for their performance by generating a standard curve using the Eurogentec qPCR MasterMix without the EuroScript, and with cDNA, which has been obtained using oligo d(T) primers and / or random nonamers. Good primers and probe can be found when following the design guidelines as mentioned p.26.

If the slope of this standard curve is -3.32 your PCR is 100 % efficient.

If the PCR efficiency is not closed to 100 % we recommend to:

- check the primer design
- optimize the primer concentration
- optimize probe concentration
- optimize the temperature reaction and times
- go for a 3-step instead of a 2-step protocol

How to determine the PCR efficiency?

The slope of the standard curve can be used to determine the exponential amplification and efficiency of the PCR reaction:

$$\text{Exponential amplification} = 10^{(-1/\text{slope})}$$

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1$$

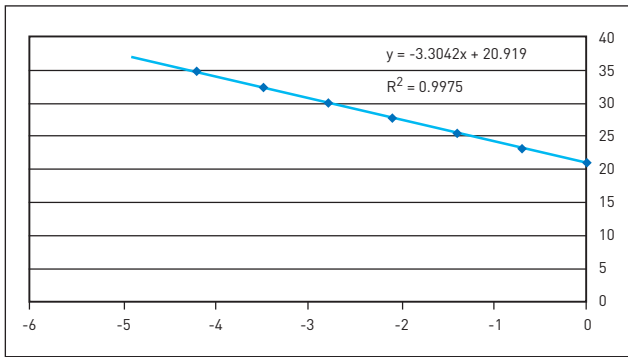


Figure 2: Standard curve made with a 10x dilution series using the One step RT qPCR MasterMix.

A 100 % efficiency corresponds to a slope of -3.32.

With 100 % efficiency, a 2x dilution gives a ΔCt of 1 between each dilution (every 1 cycle the amount is doubled).

With a 100 % efficiency, a 10x dilution gives a ΔCt of 3.2 values between each dilution (every 3.2 cycles the amount is 10 fold higher).

The R^2 is a parameter, which tells you how well the different data points lie on line. If the $R^2 < 0.95$ there is an indication that either the reactions has not been pipeted accurately or that there is no linear relation between the Ct and the log of the DNA concentration. The latter may be caused by inhibitory factors that are diluted out.

The quality of starting material plays an important role

Using poor quality RNA can limit the efficiency of the reverse transcriptase reaction and limit yields, therefore RNA extraction should always be done carefully. We recommend the use of commercially available kits to obtain good quality RNA.

Diluted solutions of RNA should be aliquoted to avoid too many freeze / thaw cycles and a carrier can be added (lamda DNA, tRNA, or BAS).

RNA samples should be stored between -20 °C and -70 °C in RNase free water (commercially available).

Diluted solutions of DNA should be aliquoted to avoid too many freeze / thaw cycles. DNA samples should be stored at -20 °C in PCR grade water (commercially available).

A standard curve should always be performed

By preparing a standard curve for every gene, which needs to be analysed, a good idea can be obtained about the performance and efficiency of the PCR. The standard curve should cover the complete range of expected expression levels (in general 6 logs of magnitude).

The standard curve gives much information on the assay (PCR efficiency, pipetting errors, and sensitivity).

How to generate a standard curve?

To plot a standard curve 6 different concentrations of the standard are normally required. These concentrations should be measured, and the amount of unknown target should fall within the range tested. The reaction should be performed in triplicate if possible.

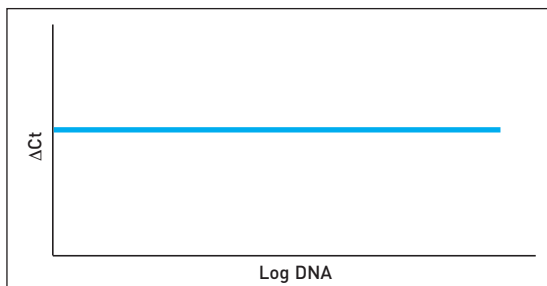
How to prepare standards?

For absolute quantification of RNA the copy number or concentration should be known. We recommend to use RNA as standards, therefore the variability of the efficiency of the reverse transcription will be taken to account. Any DNA should be removed of the standards using RNase free DNase, as this would lead into errors in the determination of RNA concentration and would also serve as template for the qPCR. After determination of the concentration, the copy number for each standard should be calculated.

How to determine the qPCR efficiency?

If the comparison of amplification efficiency of two target sequences is required, a dilution series for each of the target should be prepared. Each dilution series should be amplified by RT qPCR. Then, the Ct value of the first target should be subtracted from the Ct value of the second target. The ΔCt is then plotted against the logarithm of the template amount. If the slope is < 0.1 , the amplification efficiency is comparable.

Figure 3:
Comparable amplification qPCR
efficiencies



Multiplex negative controls

- Use a no Reverse Transcriptase control to exclude genomic DNA contamination (reaction with all the components except the reverse transcriptase).
- Use a no target control to exclude any contamination from reagents or previous PCRs (reaction with all the components except the template).
- In some cases, it could be necessary to have a positive control containing a known concentration of template to test presence or absence of the target gene and to check for inhibitory factors.

Primer and probe design

For more information about design refer to the primer design guidelines (p.26).

Protocol from sample collection to results

Sample collection

RNA degradation already starts from the first moment of sample collection. Therefore, we recommend to work in a quick way and go as fast as possible from sample to cDNA. Use of RNA-stabilizers like RNA-later (Ambion) are recommended.

RNA / DNA extraction

For the extraction of RNA from the samples, the use of an appropriate commercially available kit is recommended. There are many different kits available, which will contain all the required reagents for the full extraction / purification procedure. These kits will also outline general guidelines, such as the storage conditions and shelf life of the extracted RNA. However these guidelines may vary between kits due to the different composition of buffers. It is also recommended that the buffers supplied with each kit are used according to the protocol.

The final product should be cleaned and free from any residual buffers that may inhibit the PCR such as EDTA or solvent containing buffers. This is usually not a problem when using spin column kits instead of manual extraction techniques.

RT qPCR step

The RT reaction should be set up in a clean environment to avoid contamination; cleaning solutions are available to avoid any RNase contamination (RNASE away - Invitrogen). We also recommend to work with RNase free plastics. The tubes containing the reaction should be maintained on ice during the set-up of the reaction. This will avoid starting the reaction premature.

In a One step RT qPCR, both the RT reaction and the PCR reaction are performed in a single tube. With a One step RT qPCR only sequence specific primers can be used. These primers are the same as those used in your PCR.

The reaction is incubated at 48 °C for 30 minutes, and then the PCR is performed after the RT. The 95 °C hold required for the activation of the hotstart enzyme will also cause deactivation of the RT enzyme.

When setting up a RT qPCR reaction, it is always recommended to prepare a reaction mix, containing the primers and all the reagents required for the reaction. This minimizes differences across the 96-well plate and allows for more accurate pipetting (as the volume required per sample is usually very small).

The reaction mix should ideally be prepared in a separate room, different from the room where RNA samples have been prepared, to avoid any contamination.

The RNA samples should always be added to the side of each well before the reaction mix and then rinsed down while adding the reaction mix. As the reaction mix is heavier than the RNA, the RNA will be mixed within the reaction mix.

The plate can gently be shaken on a plate shaker and spun before placing the plate on the machine. This is not an essential step but will ensure that the reactions are thoroughly mixed and collected at the bottom of the reaction wells. It is useful to also check the wells for bubbles, as bubbles at the bottom of the well can produce unusual plots on the results, when using machines that read from the top.

The negative controls should be placed on the plate in such a position that cross contamination is avoided during the set-up; thus, they should be placed if possible away from the highest RNA concentrations, at the top or bottom of the rows to avoid going over these wells when pipeting.

When sealing the PCR plates, it is important to make sure that it has been correctly done. If optical films are used, fingerprints and marks should be avoided on the top of the film.

The qPCR machine used should be programmed according to the manufacturers instruction manual.

The ideal thermal cycling conditions can vary from assay to assay. The Technical Data Sheet supplied with the Eurogentec RT qPCR kits should be used as a good starting point, but optimization of these conditions may be required for certain assays to achieve optimal results.

Standard cycling conditions for probe assay are as follows:

	48 °C	30 min
	95 °C	10 min
40 cycles	95 °C	15 s
	60 °C	1 min

This is a 2-step PCR where the extension and annealing step are a combined 60 °C step. This is possible since the amplicons are designed to be very short and can be

copied without requiring a 72 °C extension. This protocol should be used initially when testing a new assay.

In the following cases we recommend to perform a 3-step protocol to obtain better results: if results show late Ct values, if only primer dimers are detected or if the results give a less steep growth curve and if in this special case it is not possible to redo the design.

The protocol will then be as follows:

		30 min	48 °C
40 cycles	denaturation	15 s	95 °C
	annealing	30 s	60 °C
	extension	30 s	72 °C

Extension time can be increased with 10-second steps.

It is also possible to add a data collection step after the extension step. To do so, ensure that the detection temperature is at least 3 °C lower than the T_m of the amplicon.

In addition to the cycling parameters there are some temperatures steps at the beginning of the reaction that have specific functions.

The 48 °C step for 30 minutes is used to perform the RT reaction.

The 95 °C step for 10 minutes is used to activate the hotstart *Taq* polymerase and deactivate the EuroScript. A hotstart enzyme is inactive at low temperatures (room temperature). This prevents from formation of non-specific amplification and reduces primer dimer formation. Heating at 95 °C during 10 minutes activates the enzyme and the amplification can begin once the primers are annealed.

Result analysis

All instruments are supplied with some analysis software. Instructions related to the use of this software will be contained within the manual.

Some analysis packages will allow more interaction and manipulation of the data than others, but in general case, only baseline and threshold / fit point adjustment is needed.

Few definitions

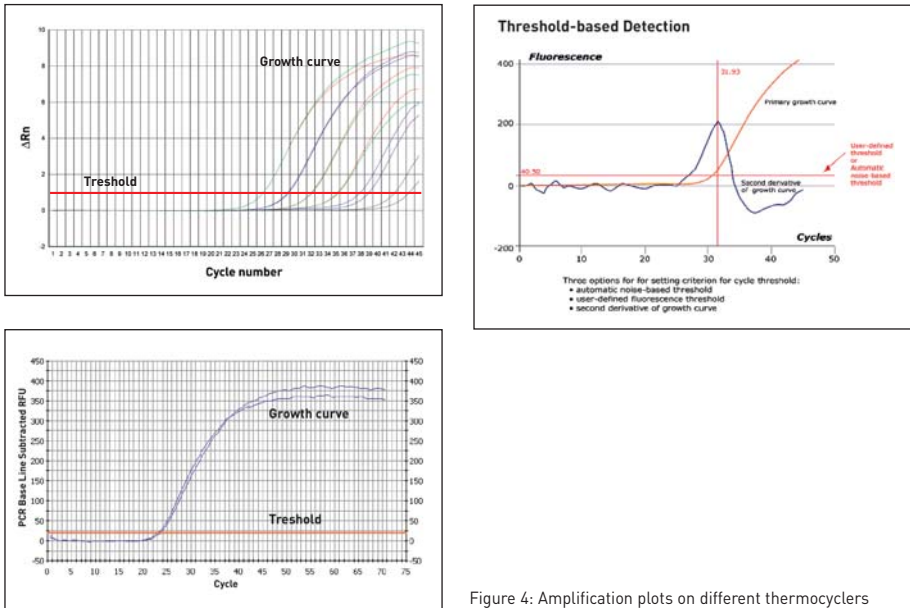


Figure 4: Amplification plots on different thermocyclers

The primary Growth Curve is the signal we usually look at. It gives the ΔRn value in case of an ABI or Mx4000® / Mx3005P® / Mx3000P®, the Rfu in case of an iCycler iQ®, My iQ®, iQ5, F1 or F2 in case of a LightCycler®.

The Second Derivative of the Growth Curve is displayed only on the Smartcycler® and calculated on the LightCycler®.

The second derivative maximum of the amplification curve determines the point at which the maximal increase in slope of the growth curve can be seen (the beginning of log linear phase). This is equivalent to the Ct value, determined via the 2nd derivative method.

The **threshold** is calculated automatically or set manually.
 If automatic: it is certain number of standard deviations above the average of the background ($\mu + x^2$).
 If manual: the threshold is adjusted to a value above the baseline, it must be located at the beginning of the log linear range of the PCR and never in the plateau phase. It corresponds to the point where all the dilution series start to be all parallel to each other.

The **Ct** is the point where the growth curve crosses the threshold and is related to the amount of initial DNA. Or the Ct is the maximum of the 2nd derivative curve. It is the first cycle in which a significant increase in signal above the background can be detected.

The Ct value shows the sensitivity of an assay: the earlier the Ct, the more sensitive the assay.

The baseline is the average background. So it corresponds to the noise level in early cycles, typically between cycle 3 and 15, when there is no detectable increase in fluorescence due to PCR products. In the most ideal case you will find a horizontal baseline. However, a slightly increasing baseline is also possible.

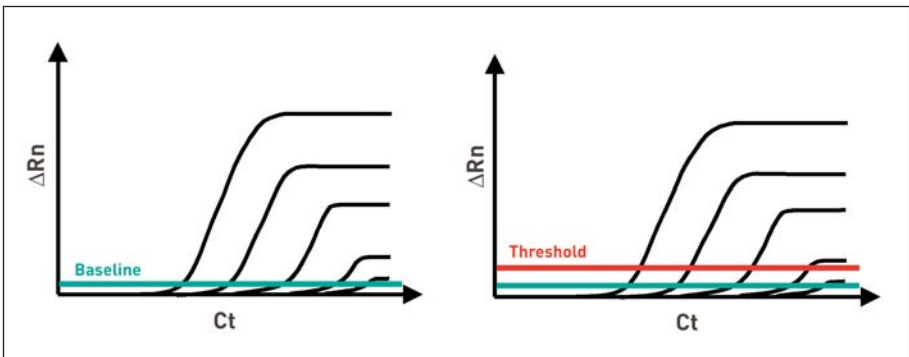


Figure 5: Amplification plots showing baseline and threshold.

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Ordering information

qPCR Core kit	500 reactions (50 µl)	RT-QP73-05
qPCR MasterMix • 5 X 1.5 ml	300 reactions (50 µl)	RT-QP2X-03
qPCR MasterMix Plus • 7.5 ml	300 reactions (50 µl)	RT-QP2X-03-075+
qPCR MasterMix Plus • 15 ml	600 reactions (50 µl)	RT-QP2X-03-15+
qPCR MasterMix Plus • 50 ml	2000 reactions (50 µl)	RT-QP2X-03-50+
qPCR MasterMix Plus w/o UNG	300 reactions (50 µl)	RT-QP2X-03WOU+
qPCR MasterMix Plus dNTP	300 reactions (50 µl)	RT-QP2X-03+WOUN
<hr/>		
qPCR Core kit for SYBR® Green I	500 reactions (50 µl)	RT-SN10-05
qPCR MasterMix for SYBR® Green I • 5 X 1.5 ml	300 reactions (50 µl)	RT-SN2X-03T
qPCR MasterMix Plus for SYBR® Green I • 7.5 ml	300 reactions (50 µl)	RT-SN2X-03+
qPCR MasterMix Plus for SYBR® Green I • 15 ml	600 reactions (50 µl)	RT-SN2X-06+
qPCR MasterMix Plus for SYBR® Green I • 50 ml	2000 reactions (50 µl)	RT-SN2X-20+
qPCR MasterMix Plus for SYBR® Green I w/o UNG	300 reactions (50 µl)	RT-SN2X-03WOU+
qPCR MasterMix Plus for SYBR® Green I dNTP	300 reactions (50 µl)	RT-SN2X-03+WOUN
<hr/>		
One step RT qPCR MasterMix	300 reactions (50µl)	RT-QPRT-032X
Two step RT qPCR Core kit	500 reactions (50 µl)	RT-QPRT-05
Two step RT qPCR MasterMix	300 reactions (50 µl)	RT-QP2X-03RT
<hr/>		
One step RT qPCR MasterMix for SYBR® Green I	300 reactions (50µl)	RT-SNRT-032X
Two step RT qPCR Core kit for SYBR® Green I	500 reactions (50 µl)	RT-SNRT-05
Two step RT qPCR MasterMix for SYBR® Green I	300 reactions (50 µl)	RT-SN2X-03RT

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