EUROGENTEC

EGT GROUP

Troubleshooting Guide

qPCR and RT qPCR kits

January 2004

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1. Introduction

Real-Time PCR is a method which has been introduced only a few years ago and has taken a flight in only a very short period of time.
The technology combines DNA amplification with real-time detection of the products in a single closed tube.
The method is very sensitive and therefore many, mainly minor, difficulties can be seen.
This troubleshooting guide takes you through the most common difficulties that you can meet when performing qPCR or RT qPCR assays using probes or SYBR® green I.
Eurogentec has a team of knowledgeable scientists who can help you solving your difficulties.
If this troubleshooting guide does not offer a solution to the difficulty you are facing feel free to contact us.

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2. Growth curve – Ct / crossing point

2.1. No Ct value…

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).

Primers or probe degraded

Check for degradation of primers and probes via denaturing PAGE.

Insufficient amount of starting DNA template

Start with a high concentration and make dilution series if the concentration is unknown. The recommended maximum amount of template is 500 ng genomic DNA.

Template DNA degraded

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

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 primer/probe Tm, 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 Tm of the probes should be 8 - 10 °C above the Tm of the primers. If the Tm 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 hybridise in the present conditions.

ΔTm between forward and reverse primer is too high

If no amplification occurs and if everything else has been excluded as cause of this result check ΔTm between forward and reverse primers. If ΔTm is more than 4 °C the PCR will not perform well or not at all.

For SYBR® green I assay only

SYBR® green I has been diluted in a watery solution

SYBR® green I is sensitive to hydrolysis when diluted in a watery solution, it will bleach if it is not kept in the dark.
Therefore, Eurogentec offers kits that contain DMSO to stabilize the SYBR® green I. Kits should be stored in the dark.

ΔTm between forward and reverse primer > 4 °C

If no amplification occurs and if everything else has been excluded as cause of this result check ΔTm between forward and reverse primers. If ΔTm is more than 4°C the PCR will not perform well or not at all.

For RT qPCR only

RT reaction temperature

We recommend performing 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.

Incorrect ratio between reverse transcriptase and PCR components

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

For One step RT qPCR MasterMix only

UNG has been added to a One step kit.

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

For One step RT qPCR MasterMix for SYBR® green I only

Insufficient amount of starting template

Increase the amount of starting material, if possible.
Typical starting concentrations are 10 pg – 50 pg of total RNA.

2.2. 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 design is not optimal or primers have been designed containing secondary structures.
If possible, we recommend redoing primer design according to usual primer design guidelines. If not, try a 3-step protocol as described (p.11), this could enhance the PCR efficiency.

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.

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

	1 Forward		
2 Reverse	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

Primers or probe degraded

Check for degradation of primers and probe via denaturing PAGE.

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	125 nM	250 nM
Opt. Primers	50/opt	125/opt	250/opt

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 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.
Dye labeled 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 hydrolyze. This will generate a low fluorescence signal and a high background (lower ΔRn).
Eurogentec recommends resuspending qPCR probes in TE 0.01 pH 8.0 instead of water.

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 Tm, 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 Tm of the probes should be 8 – 10 °C above the Tm of the primers

Template DNA degraded

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

Insufficient amount of starting DNA template

Adjust the concentration and make dilution series if the concentration is unknown. The recommended maximum amount of DNA template is 500 ng of genomic DNA (a too high concentration of DNA would give a very early Ct value – before Ct=10 – and a non linear standard curve).

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 (the size of the amplicon should not exceed 500 bp).

$MgCl_2$ concentration sub-optimal

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

Pipeting errors

As RT qPCR is a highly sensitive tool, errors will also be amplified easily.
The use of MasterMixes can reduce this, 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).
Check for PCR efficiency and for pipeting errors.

For SYBR® green I only

Primer design sub-optimal

Check via gel electrophoresis or melt curve 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 primer Tm, complementarity, secondary structures, amplicon size, number of identical nucleotides, etc…
When studying expression profiles in eukaryotic targets: choose PCR primers that span at least one exon-exon junction in the mRNA target to prevent amplification of the target from contaminating genomic DNA.
For further information follow the usual primer design guidelines.

3-step protocol instead of 2-step protocol

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

Your protocol will then be as follows:

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

Increase extension time with 10-second steps.

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

SYBR® green I has been diluted in a watery solution

SYBR® green I is sensitive to hydrolysis when diluted in a watery solution, it will bleach if it is not kept in the dark.

Therefore, Eurogentec offers kits that contain DMSO to stabilize the SYBR® green I. Kits should be stored in the dark.

For RT qPCR only

RT reaction temperature

We recommend performing 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.

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. Always prepare fresh serial dilution series of the RNA stock. Do not freeze/thaw diluted RNA especially low concentrations, as the concentration will decrease.

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

Incorrect ratio between reverse transcriptase and PCR components

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

For One step RT qPCR MasterMix for SYBR® green I only

3-step protocol instead of 2-step protocol

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

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

Increase extension time with 10-second steps.

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 Tm of the primers.

Table 1. Primer optimization matrix

	Reverse				
Forward	900 nM	600 nM	300 nM	150 nM	50 nM
900 nM	900/900	900/600	900/300	900/150	900/50
600 nM	600/900	600/600	600/300	600/150	600/50
300 nM	300/900	300/600	300/300	300/150	300/50
150 nM	150/900	150/600	150/300	150/150	150/50
50 nM	50/900	50/600	50/300	50/150	50/50

Insufficient amount of starting template

Increase the amount of starting material, if possible.
Typical starting concentrations are 10 pg – 50 pg of total RNA.

2.3. No linearity in the Ct values of a dilution series

Secondary structures in probes

For a 2x dilution series a ΔCt of 1 cycle should 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.

For One step RT qPCR for SYBR® green I

Template amount is too high.

Do not exceed maximum recommended amounts of RNA. We recommend using less than 400 pg total RNA, final concentration, per reaction.

Incorrect ratio between reverse transcriptase and PCR components

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

3. Only primer-dimers are detected

All the curves of the dilution series come up at the same Ct

This is usually caused by primer-dimers.

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 Tm, 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 Tm of the probes should be 8 - 10 °C above the Tm of the primers

$MgCl_2$ concentration too high

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

For SYBR® green I assay only

Primer design sub-optimal

Check via gel electrophoresis or melt curve 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 primer Tm, complementarity, secondary structures, amplicon size, number of identical nucleotides, etc…
When studying expression profiles in eukaryotic targets: choose PCR primers that span at least one exon-exon junction in the mRNA target to prevent amplification of the target from contaminating genomic DNA.
For further information follow the usual primer design guidelines.

3-step protocol instead of 2-step protocol

If it is not possible to redo the design, try a 3-step protocol as described p.7, this could help to obtain better results.
Your protocol will then be 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.
It is also possible to add a data collection step after the extention step. To do so, ensure that the detection temperature is at least 3 °C lower than the Tm of the amplicon.

4. Growth curve – ΔRn / fluorescence

4.1. 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/extention temperature in steps of 2 °C.

Probes hydrolysis

When probes are dissolved in an acid solution the fluorophores can hydrolyze and will give a low fluorescence signal and a high background (lower ΔRn).
Eurogentec recommends resuspending qPCR probes in TE 0.01 pH 8.0 instead of water.

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

	3 Forward		
4 Reverse	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	125 nM	250 nM
Opt. Primers	50/opt	125/opt	250/opt

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

$MgCl_2$ concentration sub-optimal

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

Pipeting 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 pipeting errors.

For SYBR® green I assay only

Low SYBR® green I concentration

Increasing the SYBR® green I concentration will increase the signal. Concentrations in the range of 1:66000 – 1:20000 will work.

SYBR® green I has been diluted in a watery solution

SYBR® green I is sensitive to hydrolysis when diluted in a watery solution, it will bleach if it is not kept in the dark. Therefore, Eurogentec offers kits that contain DMSO to stabilize the SYBR® green I. Kits should be stored in the dark.

For RT qPCR only

Incorrect ratio between reverse transcriptase and PCR components

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

Insufficient amount of starting template

Increase the amount of starting material, if possible.
Typical starting concentrations are 10 pg – 50 pg of total RNA.

For Lithos kits only (curves can sometimes be less steep and less high)

Initial denaturation step too short

HotGoldStar® DNA polymerase is a hotstart *Taq* polymerase that needs an initial activation of 10 minutes at 95 °C. Decreasing the initial activation time will lead to not complete activation of the polymerase.
In case a stairlike amplification curve is seen an improvement can be seen if the initial denaturation time is increased to 15 minutes.

Denaturation time is too short

On a fast Real-Time thermocycler denaturation times of 0 second can be used.
To amplify long amplicons an increase up to 30 seconds can also improve the results.

4.2. Less steep growth curve

PCR annealing/extension time too short

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

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

	5 Forward		
6 Reverse	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	125 nM	250 nM
Opt. Primers	50/opt	125/opt	250/opt

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 (the size of the amplicon should not exceed 500 bp).

For One step qPCR SYBR® green I only

3-step protocol instead of 2-step protocol

If your PCR efficiency is still not good after playing around annealing/extension time 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.

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 Tm of the primers.

For Lithos kits only (curves can sometimes be less steep and less high)

Initial denaturation step too short

HotGoldStar DNA polymerase is a hotstart *Taq* polymerase that needs an initial activation of 10 minutes at 95 °C. Decreasing the initial activation time will lead to not complete activation of the polymerase.
In case a stair like amplification curve is seen an improvement can be seen if the initial denaturation time is increased to 15 minutes.

Denaturation time is too short

On a fast Real-Time thermocycler denaturation times of 0 second can be used.
To amplify long amplicons an increase up to 30 seconds can also improve the results.

5. 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.

Detection of primer-dimers (negative control appears positive after the 35th cycle)

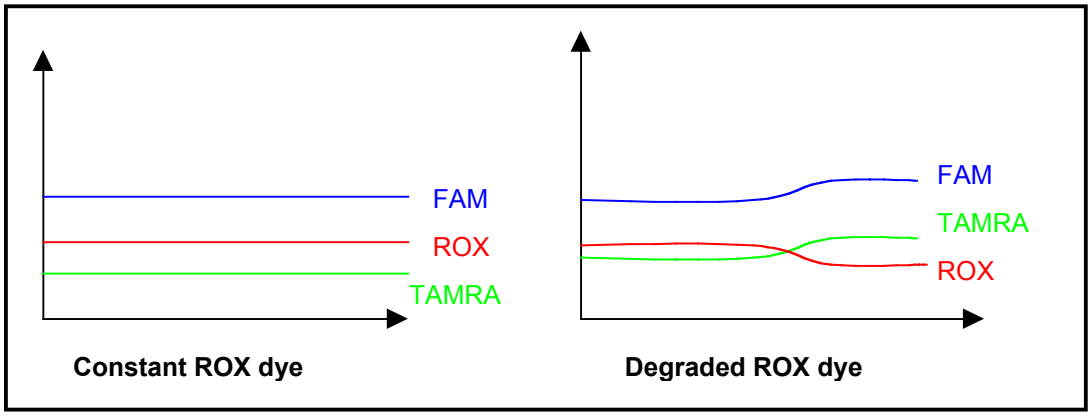
On the ΔRn graph a negative control is perfect if you get a horizontal line. But most of the time the line goes up after the 35th cycle. It means that primer dimers are detected; the *Taq* polymerase starts to process the primers.
On the melt curve graph, primer-dimers are seen around 50 – 60 °C.
On an agarose gel no product of the expected length is detected.

Probe has degraded because of heating

The signal will increase.
Run an agarose gel; if there is no product on the gel, 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 any recombinant *Taq* some traces of bacterial DNA can be found as it is produced in and purified from *E. coli*. You will have to determine the minimum level and subtract it from the positive signal. Everything above this signal should be considered as positive.

6. High fluorescence in no reverse transcriptase control

Contamination with genomic DNA

In general, it is very difficult to completely eliminate genomic DNA during the RNA purification process.

To avoid contamination, treat the RNA with RNase free DNase and design primers, which are intron spanning to make sure amplification of only cDNA (see figure p.15 and follow the usual primer design guidelines).

7. Melt curve (more than one peak visible in the meltcurve)

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

	7 Forward		
8 Reverse	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

Primers and probe design sub-optimal

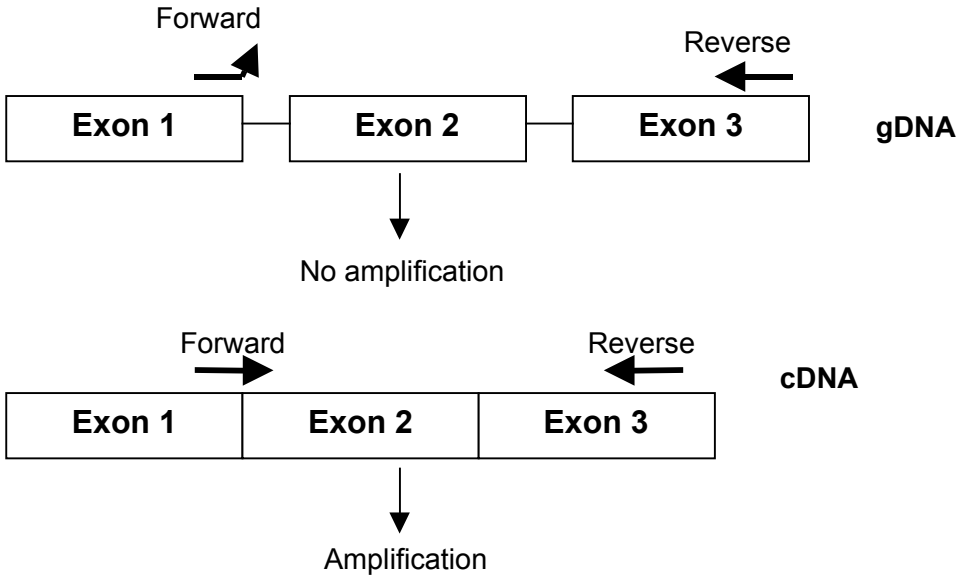
Check via gel electrophoresis for presence of any PCR product.
If no specific products can be detected redo the design. To do so, use primer design software that checks for primers/probe Tm, complementarity, secondary structures, amplicon size, number of identical nucleotides, etc…
When studying expression 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 Tm of the probes should be 8 – 10 °C above the Tm of the primers.

$MgCl_2$ concentration

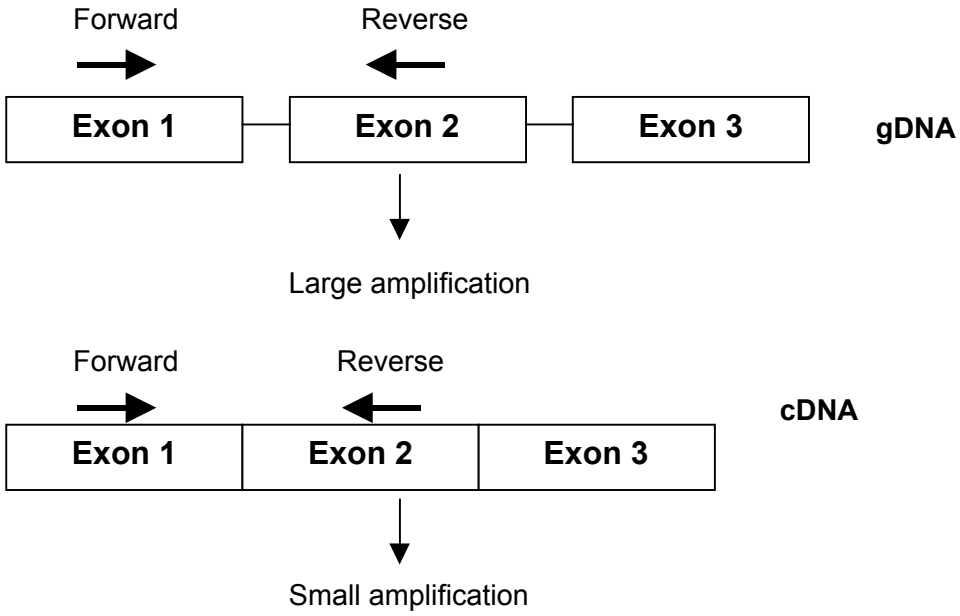
Always start with the concentration recommended in the Technical Data Sheet. For some targets an increase of $MgCl_2$ concentration can improve the results. Perform titration 0.5 mM. Always optimize primer concentration before optimizing $MgCl_2$ concentration.

Contamination with genomic DNA (follow the usual primer design guidelines)

Prepare the starting RNA template with DNase and design primers with intron spanning as shown below.



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



For SYBR® green I assay only

Primer design sub-optimal

Check via gel electrophoresis or melt curve 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 primer Tm, complementarity, secondary structures, amplicon size, number of identical nucleotides, etc…
When studying expression profiles in eukaryotic targets: choose PCR primers that span at least one exon-exon junction in the mRNA target to prevent amplification of the target from contaminating genomic DNA.
For further information follow the usual primer design guidelines.

3-step protocol instead of 2-step protocol

If it is not possible to redo the design, try a 3-step protocol as described p.7, this could help to obtain better results.
Your protocol will then be 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.
It is also possible to add a data collection step after the extention step. To do so, ensure that the detection temperature is at least 3 °C lower than the Tm of the amplicon.

For RT qPCR only

RT reaction set up done at room temperature

Set up reaction on ice to avoid premature cDNA synthesis.

RT reaction temperature

We recommend performing 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.

For One step RT qPCR MasterMix for SYBR® green I

Too high primer concentration

Use the concentration recommended in the protocol. When optimizing the assay it is advisable to use a range of concentrations above and below the recommended concentration as mentioned below:

Forward	**Reverse**				
	900 nM	600 nM	300 nM	150 nM	50 nM
900 nM	900/900	900/600	900/300	900/150	900/50
600 nM	600/900	600/600	600/300	600/150	600/50
300 nM	300/900	300/600	300/300	300/150	300/50
150 nM	150/900	150/600	150/300	150/150	150/50
50 nM	50/900	50/600	50/300	50/150	50/50

8. Standard curve

8.1. $R^2 < 0.9$

Pipeting errors

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

Imprecise dilutions

Prepare a high concentrated stock of your DNA, aliquote it to avoid freeze/thawing and keep them at –20 °C (preferably at –80 °C).
For each Real-Time PCR prepare a fresh dilution series out of the DNA template stock solution. Don not freeze diluted DNA.

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 Tm, 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 Tm of the probes should be 8 – 10 °C above the Tm of the primers.

Probe is designed with secondary structures

For a 2x dilution series a ΔCt of 1 cycle should seen between each amplification plot on 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.

For RT qPCR only

Poor quality of the starting template

Check concentration, storage and purity conditions. Always prepare fresh dilution series of the RNA stock. Do not freeze/Thaw diluted RNA especially low concentrations, as the concentration will decrease.
Use commercially available RNA extraction kits to obtain good quality RNA.
If any inhibitory components are present in the RNA, this should decrease when increasing the dilution factor.

8.2. 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 guidelines.

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 (the size of the amplicon should not exceed 500 bp).

8.3. Slope > - 3.32

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.

8.4. Non linear standard curve

Secondary structures in the primers, probe or amplicon

Refer to “primer design sub optimal”
For further information follow the usual primer design guidelines.

Probe are designed with secondary structures

For a 2x dilution series a ΔCt of 1 cycle should seen between each amplification plot on 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.

Insufficient amount of starting DNA template

Adjust the concentration and make dilution series if the concentration is unknown. The recommended maximum amount of DNA template is 500 ng of genomic DNA (a too high concentration of DNA would give a very early Ct value – before Ct=10 – and a non linear standard curve).

Inhibitors in DNA

Prepare more diluted samples, to dilute out the inhibitor.

For One step RT qPCR MasterMix for SYBR® green I

Template amount is too high

Do not exceed maximum recommended amounts of RNA. We recommend using less than 400 pg total RNA final concentration per reaction.

9. Primer and probe 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's 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 do recommend in general using a design software (for example Oligo® Primer Analysis Software) to check for all following criteria.

9.1. Primer design guidelines for SYBR® green I assays

As SYBR® green I binds to any dsDNA it is important to avoid primer-dimers and/or non-specific products in SYBR® green I assays. This can only be avoided by carefully selecting primers that only bind to the selected target. By selecting amplicons between 100 and 150 bp a high level of fluorescence can be obtained without compromising the PCR efficiency.

Primers

- length
  - 18 - 30 bases
- GC content
  - 30 – 80 % (ideally 40 – 60 %)
- Tm
  - 63 – 67 ºC (ideally 64 ºC)(Tm=2(number A+T)+4(number G+C)), so that Tannealing is 58 – 62 ºC (ideally 59 ºC)
  - ΔTm forward primer and reverse primer ≤ 4 ºC
- 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® Primer Analysis Software)
- 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® Primer Analysis Software)
- design intron spanning or intron flanking primers to prevent or identify amplification of contaminating genomic DNA (see figure p.24). 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 intron, will be smaller than the amplicons from gDNA, which will contain the intron. 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 (Vandesompele, 2002).
- Check if primers are unique and specific (check with BLAST: www.ncbi.nlm.nih.gov/BLAST/).

Amplicon

- length
  - 80 - 150 bp
  - shorter amplicons will give higher PCR efficiencies
  - longer amplicons will give a higher ΔRn as more SYBR® green I is incorporated
- GC content
  - 30 – 80 % (ideally 40 – 60 %)
- avoid secundary structures in the amplicon (check with Mfold: www.bioinfo.rpi.edu/applications/mfold/).

9.2. Primer design guidelines for probe assays

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 gives better signal to noise ratios than TAMRA. In general, we 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, YakimaYellow®, 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
  - lengths over 30 bases are possible, but it is recommended to position the quencher not at the 3' end, but internally 18 - 25 bases from the 5' end
- GC content
  - 30 – 80 %
- Tm
  - Tm of the probe must be 8 – 10 °C (8 °C for genotyping, 10 °C for expression profiling) higher than the Tm 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 mismatched 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 Tm

Primers

- length
  - 18 - 30 bases
- GC content
  - 30 – 80 % (ideally 40 – 60 %)
- Tm
  - 63 – 67 ºC (ideally 64 ºC)(Tm=2(number A+T)+4(number G+C)), so that Tannealing is 58 – 62 ºC (ideally 59 ºC)
  - ΔTm forward primer and reverse primer ≤ 4 ºC
- 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® Primer Analysis Software)
- 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® Primer Analysis Software)

- design intron spanning or intron flanking primers to prevent or identify amplification of contaminating genomic DNA (see figure p.24) 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 intron, will be smaller than the amplicons from gDNA, which will contain the intron. 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 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 secundary structures in the amplicon (check with Mfold: www.bioinfo.rpi.edu/applications/mfold/).

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