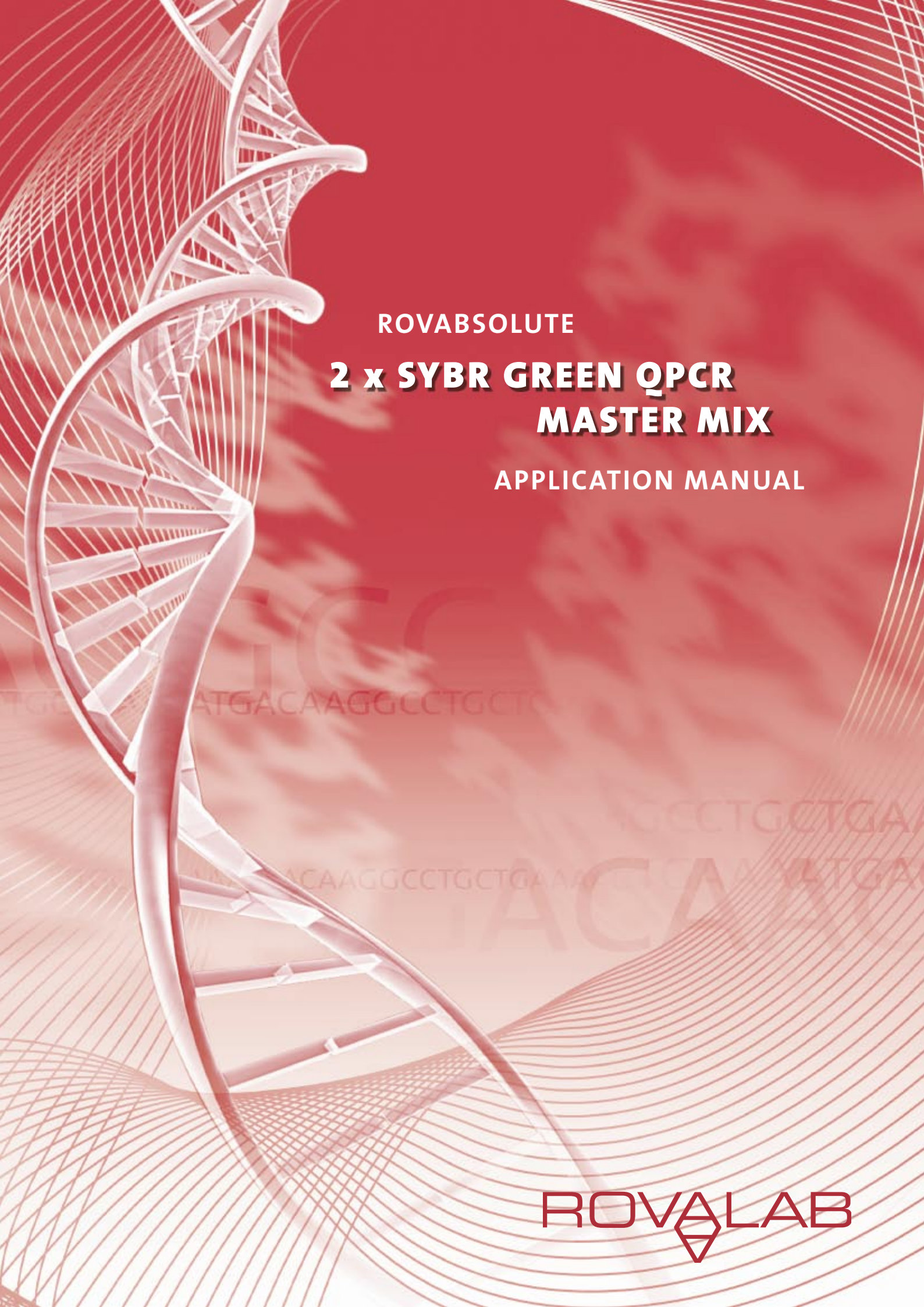


ROVABSOLUTE

2 x SYBR GREEN QPCR MASTER MIX

APPLICATION MANUAL

ROVALAB

ROVALAB

Content

ORDERING INFORMATION

Catalog #	50 µl Reactions	2 x SYBR Green Master Mix	ROX 5 µM	Fluorescein 500 nM
S 50	50	1.25 ml	250 µl	100 µl
S 200	200	4 x 1.25 ml	1000 µl	400 µl
S 400	400	8 x 1.25 ml	2000 µl	800 µl
S 2000	2000	50 ml	10000 µl	4000 µl

Please add on your order the type of reference dye nedeed : ROX or Fluorescein

RELATED PRODUCTS: QPCR Probe Mixes

Catalog #	50 µl Reactions	2 x Hot Start QPCR Master Mix	ROX 5 µM	Fluorescein 500 nM
HS 50	50	1.25 ml	250 µl	100 µl
HS 200	200	4 x 1.25 ml	1000 µl	400 µl
HS 400	400	8 x 1.25 ml	2000 µl	800 µl
HS 2000	2000	50 ml	10000 µl	4000 µl

USAGE INFORMATION

The 2 x SYBR Green Master Mix contains all reagents required for Real-Time PCR and is designed to make PCR as easy and simple as possible. All components (inclusive ROVABSOLUTE Hot Start Taq DNA-Polymerase) are provided in an optimized concentration in the 2 x SYBR Green Master solution. With 2 x SYBR Green Master Mix all you need to do is to add primers and template DNA, thus minimizing the pipetting effort and possible sources of error.

SYBR GREEN I DYE

SYBR Green I binds with a high affinity to the minor groove of double stranded dsDNA. The resulting DNA-fluorescence -complex absorbs blue light at 498 nm and emits green light at 522 nm.
During the PCR elongation step the fluorescence increases and achieves the maximum at the end of this step. SYBR Green I is then fully incorporated in double stranded DNA. During denaturation step the DNA melts and the fluorescence dye is released. Only a background signal is detectable.

REFERENCE DYES

The passive ROX reference dye has an excitation maximum at 584 nm and an emission maximum at 612 nm. The addition of the reference dye to the reaction mixture is auxiliary and its concentration has to be individually adjusted to the real time PCR instrument. ROX is photosensitive - store it in the dark whenever you can.

The Fluorescein reference dye has an excitation maximum at 494 nm and an emission maximum at 525 nm. The starting point of optimization is 50 nM Fluorescein. Optimal results are obtained between 10 - 100 nM Fluorescein.

Real-Time PCR

THEORETICAL NOTES

Quantitative Real-Time PCR assays incorporate the ability to directly monitor and quantify the reaction while amplification is taking place. Data is collected throughout the PCR process rather than at the end of the PCR process. In real-time PCR, reactions are characterized by the point in time during cycling when amplification of a target achieves a fixed level of fluorescence, rather than the amount of PCR product accumulated after a fixed number of cycles that were performed.
In a Real-Time assay, the fluorescence intensity of the reporter dye (RFU, relative fluorescent units) after each cycle is recorded and normalized to the fluorescence intensity of a passive reference dye .

METHODS

There are two methods, both equally valid, for analyzing data obtained from Real-Time PCR: Relative Standard Curve Method and Comparative C_t Method. The first, relative standard curve method, is useful for investigators that have a limited number of cDNA samples and a large number of genes of interest. The comparative C_t method is useful for investigators who have a large number of cDNA samples and a limited number of genes of interest.

CHARACTERISTIC PARAMETERS

C_t-VALUE

The threshold cycle (C_t) indicates the fractional cycle number at which the amount of amplified copies reaches a fixed threshold.

CHARACTERISTIC PARAMETERS

PCR EFFICIENCY

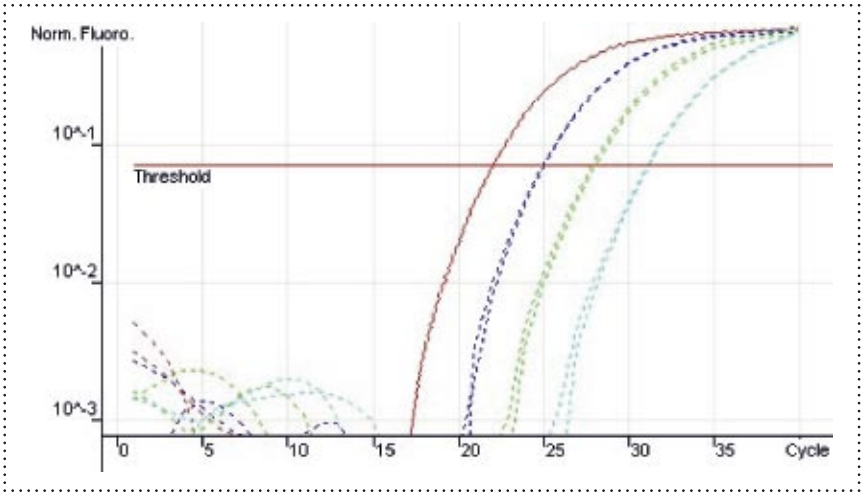
There are different methods to calculate the PCR efficiency. Their results are slightly different. A simple equation is following:

$E = 10^{-1/m} - 1$
E = efficiency
m = slope of standard curve

In "perfect" reactions, the number of amplicons doubles at each cycle and the characteristics of the resulting standard curve are:

- PCR Efficiency = 100 %
- Correlation Coefficient = 1.000
- Slope = - 3.300

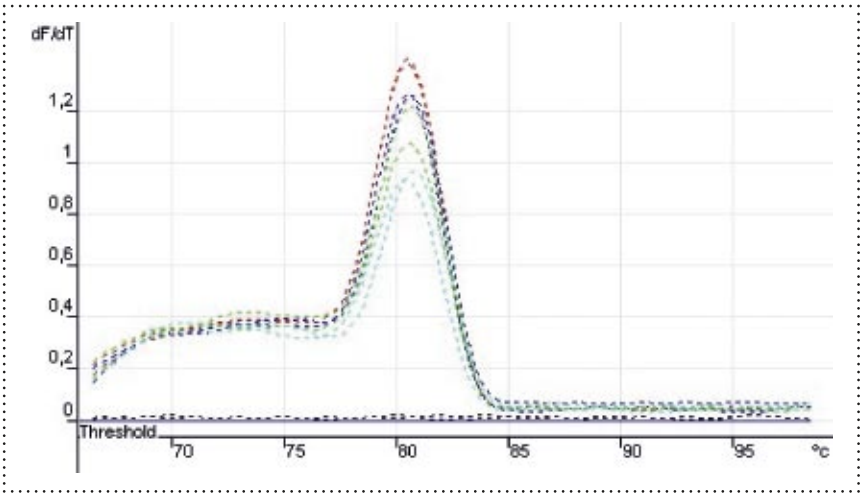
For instance, a slope of -3.58 stands for only 90 % PCR effiency.



Efficiency of the amplification of human GAPDH using log fold serial dilutions from 1 ng to 1 pg on the RG-3000 (Corbett Research).

MELTING CURVE

For SYBR Green based amplicon detection, it is important to run a dissociation curve following the real time PCR. This is due to the fact that SYBR Green will detect any double stranded DNA including primer dimers, contaminating DNA, and PCR product from misannealed primer.
The Tm of the amplicon starts at the point of inflection of the melting curve profile.



Derivative Melting Curve for Standard Curve Samples in Real Time, GAPDH Endogeneous Control

It is apparent that the point of inflection (melting temperature of the amplicon) occurs at 81.5 °C. Also we can see that no contaminating products are present in this reaction. Contaminating DNA or primer dimers would show up as an additional peak separate from the desired amplicon peak.

PCR OPTIMIZATION

- Use sterile technique and always wear fresh gloves when working in the PCR area. Change gloves frequently, especially if you suspect they have become contaminated with solutions containing template DNA.
- Always use new and/or sterilized glassware, plasticware, and pipettes to prepare PCR reagents and template DNA.
- Autoclave all reagents and solutions that can be autoclaved without affecting their performance. Of course, primers, layer of mineral oil, dNTPs and Taq DNA Polymerase should not be autoclaved.
- Have your own set of PCR reagents and solutions that are used only for PCR. Store these reagents in small aliquots.
- When pipetting DNA, avoid creating aerosols that could carry contaminants. Always include control reactions, for example a negative ("no DNA") control which contains all reaction components except the template DNA, and a positive control that has been successfully used in previous PCRs.

The polymerase chain reaction (PCR) yields different results as a function of primer, target DNA, buffer and salt concentrations and pH. To determine the optimal conditions for your specific PCR application and number of reaction component concentrations should be varied to provide the best amplification product. The following characteristics are suggested:

TEMPLATE

The template should be of high molecular weight and its purity and integrity checked on an agarose gel prior to use. Contamination of the DNA template with RNA can result in reduced yield in PCR due to chelation of Mg^{2+} and hence should be prepurified. Template concentration will have a profound effect on PCR performance. Recommended amounts for standard PCR and reaction volume 25 µl are:

- Human DNA up to 500 ng
- Plasmid DNA 0.1–10 ng
- Bacterial DNA 1–10 ng

PRIMERS

The influence of primer selection is critical to successful PCR, and is a function of sequence and concentration. Optimum primers work because:

- > they are 18–24 bases long.
- > contain no internal secondary structure.
- > contain 40–60% G/C.
- > have a balanced distribution of G/C and A/T rich domains.
- > are not complementary to each other at the 3‘ ends to avoide primer-primer dimer formation.
- > have a melting temperature (Tm) that allows an nealing temperatures of 53°–68°C (for maximum specificity use temperatures of 62°–65°C). Optimum annealing temperatures must determined empirically.
- > Primer concentrations between 0.1 and 0.6 µM are generally optImal.

Higher primer concentrations may promote mispriming and accumulation of non¬specific product. Lower primer concentrations may be exhausted before the reaction is completed, resulting in lower yields of the desired product.

ANNEALING TEMPERATURE

For primers with length > 13 nucleotides:

Simple method:

$$T_m = 2°C \times (A + T) + 4°C \times (G + C)$$

GC“ method:

$$T_m = 64 + \frac{G + C - 16.4}{A + G + C + T}$$

Salt-adjusted“ method

$$T_m = 100.5 + 41 \times \frac{C + G}{A + C + G + T} - \frac{820}{A + C + G + T} \times 16.6 \times \log_{10}([Na^+])$$

[Na+] = concentration of sodium ions

$MgCl_2$ CONCENTRATION

Mg^{2+} forms soluble complexes with dNTPs to produce the actual substrate that the polymerase recognizes. The concentration of free Mg^{2+} depends on the concentrations of compounds that bind the ion, including dNTP, free pyrophosphate (PPi) and EDTA.
For best results, always determine the optimal $MgCl_2$ concentration empirically. The optimal $MgCl_2$ concentration may vary from approximately 1.5 mM to 2.5 mM. The most commonly used $MgCl_2$ concentration is 1.5 mM (with dNTPs at a concentration of 200 µM each). Mg^{2+} influences enzyme activity and increases the Tm of double-stranded DNA. Excess Mg^{2+} in the reaction can increase non-specific primer binding and increase the non-specific background of the reaction.

Magnesium chloride solutions form a concentration gradient when frozen and need to be vortexed prior to use.

REACTION ADDITIVES

In some cases, adding the following compounds can enhance the efficiency or specificity of PCR:

> Betaine (0.5–2 M)
> Bovine serum albumin (BSA; 100 ng/50 µl)
> Detergents
> Dimethylsulfoxide
> Gelatine
> Pyrophosphatase (0.001–0.1 units/reaction)
> Spermidine
> T4 Gene 32 protein

ADDTITIVE YOU SHOULD NOT MIX IN THE PCR REACTION

Some impurities have a strong inhibitory effect on PCR

Impuritity	Concentration
SDS	> 0.005 % (w/v
Ethanol	> 1 % (v/v)
Phenol	> 0.2 % (v/v)
Sodium acetate	> 5 mM
Isopropanol	> 1 % (v/v)
EDTA	> 0.5 mM
Sodium chloride	> 25 mM

Make sure you know how you got your source DNA. Chloroform, phenol, EDTA, ionic detergents (SDS and Sarkosyl), xylene cyanol, bromophenol blue and ethanol among many other things can inhibit PCR. An extra clean-up step on your template may do the trick. Also, certain polymerases can be more susceptible to certain substances, so be sure to check your polymerase for possible inhibitors.

DIFFICULT TEMPLATES

For better amplification results use additional substances for example glycerol (5 – 10 %), DMSO (2 – 20 %), formamide (2 – 20 %), tetramethylammonium chloride (0.01 – 10 mM) or combinations of these.
The role of DMSO and glycerol can significantly reduce the presence of non-specific binding products, which results in band smearing and presence of non-target sequence products.

PROTOCOLS

BASIC PROTOCOL

Combine the following components in a PCR-reaction tube and adjust to a final volume of 25 µl with PCR grade H2O:

Component	Concentration	Volume	Final concentration
2 x SYBR Green Master MiX	2 x	12.5 µl	1 x
Primer A	1 µM	variable	0.2 - 1 µM
Primer B	1 µM	variable	0.2 - 1 µM
Template DNA	10 ng / µl	variable	1 – 150 ng*1
PCR grade H_2O		variable	
Final volume		25 µl	

*1: 10 ng/25 µl reaction for linearized plasmid DNA
50 ng/25 µl – 125 ng/25 µl for mammalian genomic DNA

Total volume of 50 µl is also possible.
Mix well, centrifuge and place in cycler. Optimal conditions for concentration of primer, template and MgCl2 and temperature profile need to be determined for each reaction.

Cycling Conditions:

Temperature	Step	Time	Number of cycles
92 °C - 94 °C	initial denaturation	3 min – 5 min	1
94 °C	denaturation	20 sec – 1 min	35 – 45*2
53 °C – 68 °C	annealing*1	20 sec – 1 min	
72 °C	elongation	0.6 – 0.7 min / kb	
72 °C	final elongation	0.6 – 0.7 min / kb	1

*1: depends on primer
*2: 35 cycles for plasmid DNA; 40 – 45 cycles for genomic DNA

ABI PRISM 7000, 7300, 7700, 7900(HT) / GeneAmp 5700 Protocol

Reaction Mixture

Component	Concentration	Volume	Final concentration
2 x SYBR Green Master Mix	2 x	25 µl	1 x
Forward Primer	3 µM	5 µl	0.3 µM
Reverse Primer	3 µM	5 µl	0.3 µM
ROX (optional)		3 - 5 µl	300 nM - 500 nM
PCR grade H_2O		variable	
Final volume		45 µl	

45 µl of reaction mixture and 5 µl of diluted human genomic DNA were pipetted into an optical 96-well plate. The plate was covered with optical caps and centrifuged. The contents were collected in the bottom of the tubes and bubbles were removed.

Cycling Conditions

Temperature	Time	Number of cycles
95 °C	10 min	1
95 °C	30 sec	40
55 °C - 60 °C	1 min	
72 °C	1 min (short target 50-400bp) 1.5 min (long target 400-900bp)	
72 °C	3 min (long target 400-900bp)	1

CORBETT ROTOR -GENE 3000, CORBETT ROTOR -GENE 6000 Multichanel Protocol

Reaction Mixture

Component	Concentration	Volume	Final concentration
2 x SYBR Green Master Mix	2 x	25 µl	1 x
Forward Primer	5 µM	1,5 µl	0.15 µM
Reverse Primer	5 µM	1,5 µl	0.15 µM
PCR grade H_2O		18 µl	
Final volume		46 µl	

46 µl of reaction mixture and 4 µl of diluted human genomic DNA (25 µg / µl) were pipetted into an optical 96-well plate. The plate was covered with optical caps and centrifuged. The contents were collected in the bottom of the tubes and bubbles were removed.

Cycling Conditions

Temperature	Time	Number of cycles
95 °C	5 min	1
95 °C	30 sec	40
53 °C	30 sec	
72 °C	30 sec	

BIORAD iCycler, MyiQ, iQ5 Protocol

Reaction Mixture

Component	Concentration	Volume	Final concentration
2 x SYBR Green Master Mix	2 x	25 µl	1 x
Forward Primer	3 µM	5 µl	0,3 µM
Reverse Primer	3 µM	5 µl	0.3 µM
Fluorescein (optional)	100 nM*1	(5 µl)	10 nM
PCR grade H_2O		to 5 µl	
Final volume		40 µl	

*1: dilution 1:5 from stock solution

40 µl of reaction mixture and 10 µl of diluted human genomic DNA were pipetted into an optical 96-well plate. The plate was covered with optical caps and centrifuged. The contents were collected in the bottom of the tubes and bubbles were removed.

Cycling Conditions

Temperature	Time	Number of cycles
95 °C	3 min	1
95 °C	15 sec	45
62 °C	45 sec	

DNA ENGINE Opticon 2, Chromo 4

Reaction Mixture

Component	Concentration	Volume	Final concentration
2 x SYBR Green Master Mix	2 x	10 µl	1 x
Template DNA			1 - 5 ng
Forward Primer	5 µl	2 µl	500 nM
Reverse Primer	5 µl	2 µl	500 nM
ROX (optional)	500 nM *1	1.2 - 2 µl	30 - 50 nM
PCR grade H2O fill up		to 20 µl	
Final volume		20 µl	

*1: dilution 1:10 from stock solution
Total volume of 25 - 50 µl is also possible.

Cycling Conditions

Temperature	Time	Number of steps
94 °C	3 min	1
94 °C	45 sec	2
55 °C	30 sec	3
72 °C	180 sec	4
83 °C	1 sec	5
	Plate read	6
	Go to step 2, 40 - 45 more times	7
72 °C	10 sec	8
10 °C	Forever	9
	END	

STRATAGENE MX3000P, MX3005P AND MX4000, ABI 7500 PROTOCOL

Reaction Mixture

Component	Concentration	Volume	Final concentration
2 x SYBR Green Master Mix	2 x	12.5 µl	1 x
ROX (optional)	500 nM *1	1.5 –2.5 µl	30 nM - 50 nM
Forward Primer			50-150 nM
Reverse Primer			50-150 nM
PCR grade H_2O fill up		to 25 µl	
Final volume		25 µl	

*1: dilution 1:10 from stock solution.
Total volume of 50 µl is also possible.

Cycling Conditions

Temperature	Time	Number of cycles
95 °C	10 min	1
95 °C	30 sec	40
55 °C - 60 °C	1 min	
72 °C	1 min (short target 50-400bp) 1.5 min (long target 400-900bp)	
72 °C	1 min (short target) 3 min (long target)	1

ROCHE Light-Cycler Protocol

Reaction Mixture

Component	Concentration	Volume	Final concentration
2 x SYBR Green Master Mix	2 x	10 µl	1 x
Template		variable	< 500 ng
Forward Primer		variable	300 nM
Reverse Primer		variable	300 nM
PCR grade H_2O fill up		to 20 µl	
Final volume		20 µl	

Cycling Conditions

Temperature	Time	Number of cycles
95 °C	15 min	1
95 °C	15 sec	40
60 °C *1	20 sec	
72 °C	20 sec	

*1: Annealing temperature dependent on primers.

MELT CURVE Example

Temperature	Time	Number of cycles
95 °C	5 sec	1
45 °C	60 sec	1
95 °C	5 sec	80
40 °C	60 sec	1

Increase set point temperature by 0.1 °C per second.

TROUBLESHOOTING

NO AMPLIFICATION

Pipetting error / reagents missing	> Repeat experiment checking concentration of all reagents.
Enzyme not activated	> Check that the full 5 minute activation step is performed before cycling.
Annealing step	> Check that you have the optimal annealing temperature by performing a temperature gradient (2°C increments). Annealing time should be carried out as specified in the protocol.
Extension step	> Extension time can be increased for longer amplicons. > Amplification of products over 300 bp is not recommended.
Primers	> Poor primer design - Check for primer dimers on gel. > Wrong primer concentration - 0.4µM recommended. > Primers degraded - Check on polyacrylamide gel. Re-order new primers if necessary.
Detection step	> Detection reading taken at wrong step. Reading should be taken in the annealing step for Molecular Beacons or the detection/annealing step for hybridisation probes.
Product too long	> The ideal amplicon size is between 80-200bp. Amplification of products over 300 bp is not recommended.
To few cycles	> 40 cycles is recommended.

TROUBLESHOOTING

NO AMPLIFICATION

Template	> Impure template - Purify template before use. For templates isolated from difficult sources (such as plant) use a commercial mix containing enhancers/ detergents. > Wrong concentration - A concentration of up to 500 ng can usually be used. > Degraded - Make fresh dilution from stock. Check storage conditions.
Wrong dye layer	> Check that machine settings correspond with experiment.
Probes	> Poor probe synthesis - Check with rDNase I digestion. Once digested the fluorescence should increase greatly as the quencher and fluorophore become separated. Poor probe design.

WAVY / ERRATIC LINES

No ROX	> Check machine settings. ABI Prism requires ROX for normalisation. ROX is available as a separate vial.
Too many cycles performed	> Reduce number of cycles.
Wrong detection step	> Check detection step is set in the correct stage of the cycle.
Machine needs calibrating	> Wavy lines can be caused by mirror misalignment or lamp problems. Consult machine manufacturer.
Baseline for Δ Rn set at wrong cycle	> Δ Rn should be set between 3-15 cycles and at least 2 cycles before 1st dilution amplifies.
Reaction volume too low.	> Some QPCR instruments (e.g. ABI Prism 7700) are set to read accurately only at volumes of at least 15 µl.

TROUBLESHOOTING

AMPLIFICATION IN NO TEMPLATE CONTROL

Primer dimers

- Primer dimers can also be identified by using a serial dilution of your template and running products on a gel. As template concentration increases, the primer dimer bands should decrease in intensity. If the presence of primer dimers is observed, then it may help to do one of the following:
  1) re-design the primers,
  2) try increasing the annealing temperature, or
  3) decrease the primer concentration.
- If using a standard curve, a sub-optimal gradient can indicate inhibition from primer dimers.

Contamination

- Template contaminated - Purify template before use.
- If doing QRT-PCR, treat RNA template with recombinant DNase I or design exon-spanning primers.
- DNA polymerase contaminated - All recombinant DNA polymerases will contain small amounts of E. coli DNA. However, if contamination remains a problem, a "BLAST"search can be performed to check for homology to the E. coli genome.
- Reagents contaminated - Repeat with fresh reagents and always use filter tips.

HIGH WELL-TO-WELL VARIANCE

Poor plate choice

- Do not use frosted or black plates.

Low quality sealing material

- Use only high quality optically clear seals that have been specifically designed for fluorescence applications.

Machine needs re-calibrating

- Follow manufacturer's guidelines.

Evaporation

- Do not use corner wells or use a more robust seal.

Concentration gradient formed in vial

- Invert the mixture a couple of times before use.

TROUBLESHOOTING

LOW SENSITIVITY (High Cycle Threshold (Ct))

Evaporation	> Do not use corner wells or use a more robust seal.
Low quality sealing material	> Use only high quality optically clear seals that have been specifically designed for fluorescence applications.
Primer dimers	> Primer dimers can also be identified by using a serial dilution of your template and running products on a gel. As template concentration increases, the primer dimer bands should decrease in intensity. If the presence of primer dimers is observed, then it may help to do one of the following: 1) re-design the primers, 2) try increasing the annealing temperature, or 3) decrease the primer concentration. > If using a standard curve, a sub-optimal gradient can indicate inhibition from primer dimers.
Annealing step	> Check that you have the optimal annealing temperature by performing a temperature gradient (2°C increments). > Annealing time should be carried out as specified in the protocol. > Start with the recommended annealing/extension time (60 seconds) and increase with 10-seconds steps.
Extension step	> Extension time can be increased for longer amplicons. Increase annealing/extension temperature in steps of 2°C. > Amplification of products over 300 bp is not recommended.

TROUBLESHOOTING

LOW SENSITIVITY (High Cycle Threshold (Ct))

Primers

- Wrong primer concentration - 0.4µM recommended.
- To optimize use different ratios of reverse/forward primer mixes e.g. 50/50 nM, 300/50 nM, 900/50 nM, 300/50 nM, 300/300 nM ...
- Check for degradation of primers and probes via denaturing PAGE.

Efficiency is poor

- This is usually caused by the length of the amplicon. 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, this could enhance the PCR efficiency.

Probes may have bleached and hydrolyzed

- If it have been left in the light for some time. When probes are dissolved in an acid solution, the fluorophores can hydrolyze.

Pipetting errors

- As Real Time PCR is a highly sensitive tool, error will also be amplified easily. The use of Master Mixes can reduce this, as variability is kept to a minimum. A standard curve should always be used to check for irregularities. Check for PCR efficiency and pipetting errors.

NO CT VALUE

Number of cycles is insufficient

- Start with 35 cycles, and then increase up to 45 cycles. More than 45 cycles increase the background.

Detection during wrong PCR step

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

Primers or probes degraded

- Check for degradation of primers and probes via denaturing PAGE.

TROUBLESHOOTING

NO CT VALUE	
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.
Primer or 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 probes 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 betwen 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 wil not perform well or not at all.

TROUBLESHOOTING

NO LINEARTIY IN THE CT VALUES OF A DILUTION SERIES

Secondary structures in probes

- For a 2 x dilution series a ΔCt of 1 cycle should seen between each dilution in the growth curve and for a 10 x 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.

LOW Δ RN

- Annealing/extension time too short
- Annealing/extension temperature too high or to low
- Probes or SYBR Green hydrolysis
- Primer concentration or ratio sub-optimal
- Probe or SYBR Green may have bleached
- Pipetting errors

LESS STEP GROWTH CURVE

- Annealing/extension time too short
- Primer concentration or ratio sub-optimal
- PCR product too long

HIGH FLUORESCENCE IN THE NEGATIVE CONTROL

Contamination from previous PCR

- Clean work practices should be used to avoid DNA template contamination

TROUBLESHOOTING

HIGH FLUORESCENCE IN THE NEGATIVE CONTROL	
Detection of primer-dimers	> Primer dimers can also be identified by using a serial dilution of your template and running products on a gel. As template concentration increases, the primer dimer bands should decrease in intensity. If the presence of primer dimers is observed, then it may help to do one of the following: 1) re-design the primers, 2) try increasing the annealing temperature, or 3) decrease the primer concentration. > If using a standard curve, a sub-optimal gradient can indicate inhibition from primer dimers.
Probe or ROX has degraded because of heating	> Run a agarose gel. If there is no product on the gel, probes or ROX dye has degraded.
MORE THAN ONE PEAK VISIBLE IN MELTCURVE	> Primer concentration or ratio sub-optimal > Primer and probe design sub-optimal
STANDARD CURVE: R^2<0.9	> Pipetting errors > Imprecise dilutions - Prepare a high concentrated stock of your DNA, aliquote it to avoid freeze/ thawing and keep them at -20°C (preferably -80°C). For each Real Time PCR prepare a fresh dilution series out of the DNA template stock solution. Do not freeze diluted DNA. > Primer and probe design sub-optimal
STANDARD CURVE: Slope>-3.32 (<100% PCR efficiency)	> Secondary structures in primers, probe or amplicon > PCR product too long > Probe degraded

TROUBLESHOOTING

STANDARD CURVE: non-linear	› Secondary structures in primers, probes or amplicon › Insufficient amount of starting DNA template › Inhibitors in DNA - Prepare more diluted samples, to dilute out the inhibitor.
SYBR GREEN I **SYBR Green I has been diluted in a watery solution**	› SYBR green I is sensitive to hydrolysis when diluted in watery solution, it will bleach if it is not kept in the dark.

STORAGE

For long term storage freeze at –20 °C. For frequent use, storage at 4 °C is possible for two weeks. Avoid frequent freeze – thaw cycles. Aliquotation of 2 x SYBR Green Master Mix is recommended. Store protected from light as 2 x SYBR Green Master Mix and reference dye are light sensitive.

WARNING

For research use only. Do not use internally or externally in humans or animals.

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