

## IV. PCR Related Products

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## Product Overview

### DNA Polymerases

Prod.No.	Product	Pack-size	Price in €
M3185.0250	DF Taq Polymerase (DNA-free)	250 units	74.35
M3185.0500	DF Taq Polymerase (DNA-free)	2 x 250 units	109.50
M3185.1000	DF Taq Polymerase (DNA-free)	4 x 250 units	195.00
M3185.2500	DF Taq Polymerase (DNA-free)	10 x 250 units	337.50
M3000.0250	LongMax PCR Kit (up to 20kb)	250 units	186.87
M3000.0100	LongMax PCR Kit (up to 20kb)	100 units	79.08
M3000.2500	LongMax PCR Kit (up to 20kb)	5 x 500 units	1565.83
M3004.0250	<i>Pfunds</i> proof reading polymerase	250 units	75.00
M3004.0500	<i>Pfunds</i> proof reading polymerase	2 x 250 units	145.00
M3004.1250	<i>Pfunds</i> proof reading polymerase	5 x 250 units	299.00
M3002.0100	Pwo Polymerase	100 units	35.00
M3002.0500	Pwo Polymerase	2 x 250 units	155.00
M3002.1250	Pwo Polymerase	5 x 250 units	340.00
M3305.0250	RedTaq (Taq-polymerase labeled with red pigment)	250 units	75.00
M3305.0500	RedTaq (Taq-polymerase labeled with red pigment)	500 units	95.00
M3305.2500	RedTaq (Taq-polymerase labeled with red pigment)	10 x 250 units	375.00
M3003.0100	ReproFast Polymerase	100 units	42.00
M3003.0250	ReproFast Polymerase	250 units	85.00
M3003.1250	ReproFast Polymerase	5 x 250 units	335.00
M3012.0250	ReproHot Polymerase	250 units	109.50
M3012.1000	ReproHot Polymerase	1000 units	349.75
M3043.0250	Taq Polymerase E (high efficiency)	250 units	50.00
M3043.0500	Taq Polymerase E (high efficiency)	2 x 250 units	65.00
M3043.1000	Taq Polymerase E (high efficiency)	4 x 250 units	120.00
M3043.2500	Taq Polymerase E (high efficiency)	10 x 250 units	275.00
M3001.0250	Taq Polymerase S (high specificity)	250 units	50.00
M3001.0500	Taq Polymerase S (high specificity)	2 x 250 units	65.00
M3001.1000	Taq Polymerase S (high specificity)	4 x 250 units	120.00
M3001.2500	Taq Polymerase S (high specificity)	10 x 250 units	275.00
M3001.5000	Taq Polymerase S (high specificity)	20 x 250 units	525.00
M3005.0500	Tth Polymerase	500 units	103.81
M3005.2500	Tth Polymerase	5 x 500 units	412.89
M3006.0200	HotStart Taq with Antibody	200 units	70.00
M3006.1000	HotStart Taq with Antibody	1000 units	275.00
M3307.0250	SuperHot Taq-Polymerase for qPCR	250 units	90.00
M3307.1000	SuperHot Taq-Polymerase for qPCR	1000 units	275.00

## IV. PCR Related Products

### PCR Mastermixes

#### PCR Kits - Mastermixes

Prod.No.	Product	Pack-size	Price in €
M3023.0100	GreenMasterMix (2X)	2.5 mL	115.00
M3023.0500	GreenMasterMix (2X)	12.5 mL	475.00
M3011.0100	GreenMasterMix (2X) Low ROX	2.5 mL	115.00
M3011.0500	GreenMasterMix (2X) Low ROX	12.5 mL	475.00
M3052.0100	GreenMasterMix (2X) High ROX	2.5 mL	115.00
M3052.0500	GreenMasterMix (2X) High ROX	12.5 mL	475.00
M3007.0100	Hotstart Mastermix (2-times)	2.5 mL	69.50
M3007.0500	Hotstart Mastermix (2-times)	12.5 mL	312.70
M3014.0100	PCR Mastermix (2X)	2.5 mL	55.00
M3014.0500	PCR Mastermix (2X)	12.5 mL	245.00
M3029.0100	PCR RedMaster (2X)	2.5 mL	62.50
M3029.0500	PCR RedMaster (2X)	12.5 mL	295.50
M3010.0100	ProbeMasterMix (2X) High ROX	2.5 mL	115.00
M3010.0500	ProbeMasterMix (2X) High ROX	12.5 mL	475.00
M3327.1000	Taq-E PCR Kit with dNTPs	1000 units	145.00
M3327.2500	Taq-E PCR Kit with dNTPs	2500 units	295.75
M3313.1000	Taq-S PCR Kit with dNTPs	1000 units	145.00
M3313.2500	Taq-S PCR Kit with dNTPs	2500 units	295.75

### HotStart & Realtime PCR

#### HotStart Enzymes

Prod.No.	Product	Pack-size	Price in €
M3008.0050	DirectBlood Mastermix (2X)	50 rxn	145.00
M3008.0200	DirectBlood Mastermix (2X)	200 rxn	335.00
M3009.020M	HotStart Taq PCR Kit with dNTPs	200 units	78.00
M3009.100M	HotStart Taq PCR Kit with dNTPs	1000 units	290.00
M3009.100S	HotStart Taq PCR Kit with dNTPs	1000 units	290.00
M3006.0200	HotStart Taq with Antibody	200 units	70.00
M3006.1000	HotStart Taq with Antibody	1000 units	275.00
M3306.025M	SuperHot Taq PCR Kit with dNTPs	250 units,	90.00
M3306.100M	SuperHot Taq PCR Kit with dNTPs	1000 units	290.00
M3306.100S	SuperHot Taq PCR Kit with dNTPs	1000 units	290.00
M3307.0250	SuperHot Taq Polymerase for qPCR	250 units	90.00
M3307.1000	SuperHot Taq Polymerase for qPCR	1000 units	275.00

### PCR Miscellaneous

#### Buffers

Prod.No.	Product	Pack-size	Price in €
M3453.0015	10X PCR Buffer S incomplete	1.5 mL	3.75
M3454.0015	10X PCR Buffer S complete	1.5 mL	3.75
M3455.0015	10X PCR Buffer E incomplete	1.5 mL	3.75

## PCR - Miscellaneous

Prod.No.	Product	Pack-size	Price in €
M3456.001	10X PCR Buffer E complete	1.5 mL	3.75
M3318.0010	10X PCR Buffer X complete	1 mL	5.00
M3373.0250	custom made 10X PCR Buffer	250 mL	192.94
M3373.1050	custom made 10X PCR Buffer	10 x 50 mL	281.14
M3373.4250	custom made 10X PCR Buffer	4 x 250 mL	385.88
M3032.0500	B-Enhancer solution	500 µL	10.00
M3032.0005	B-Enhancer solution	5 mL	30.00
M3308.0020	DNA Loading buffer I	4 x (5 x 1 mL)	77.18
M3308.0005	DNA Loading buffer I	5 x 1 mL	22.05
M3321.0020	DNA Loading buffer II	4 x (5 x 1 mL)	77.18
M3321.0005	DNA Loading buffer II	5 x 1 mL	22.05
M3024.0810	Mineral Oil	8 x 10 mL	48.12
M3039.0150	Oligo dT20	5 OD	27.50
M3038.0125	Random-Primer N6	5 OD	27.50
M3034.0500	RNase Inhibitor	500 units	52.02
M3034.2000	RNase Inhibitor	2000 units	70.78
M3034.1010	RNase Inhibitor	10000 units	271.03
M3013.0250	Tth Inorganic Pyrophosphatase Thermostable	250 units	84.00
M3013.1000	Tth Inorganic Pyrophosphatase Thermostable	1000 units	267.75
M3096.0200	Uracil-DNA Glycosylase (UDG)	200 units	53.09
M3096.1000	Uracil-DNA Glycosylase (UDG)	1000 units	206.44

## Nucleotides

Prod.No.	Product	Pack-size	Price in €
M3436.0000	dAMP	on request	on request
M3402.0100	dATP Powder, min. 97.0%	100 mg	106.17
M3402.0250	dATP Powder, min. 97.0%	250 mg	200.54
M3402.1000	dATP Powder, min. 97.0%	1 g	589.84
M3018.0020	dATP solution	20 µmol	46.73
M3018.0100	dATP solution	100 µmol	152.72
M3437.0000	dCMP	on request	on request
M3401.0100	dCTP Powder, 96.0% to 98.0%	100 mg	106.17
M3401.0250	dCTP Powder, 96.0% to 98.0%	250 mg	200.54
M3401.1000	dCTP Powder, 96.0% to 98.0%	1 g	589.84
M3019.0020	dCTP solution	20 µmol	46.73
M3019.0100	dCTP solution	100 µmol	152.72
M3438.0000	dGMP	on request	on request
M3403.0100	dGTP Powder, min. 97.0%	100 mg	106.17
M3403.0250	dGTP Powder, min. 97.0%	250 mg	200.54
M3403.1000	dGTP Powder, min. 97.0%	1 g	589.84
M3020.0020	dGTP solution	20 µmol	46.73
M3020.0100	dGTP solution	100 µmol	152.72
M3015.4020	dNTP-Set (Na-salt) - 100 mM	4 x 20 µmol	50.00
M3015.4100	dNTP-Set (Na-salt) - 100 mM	4 x 100 µmol	175.00
M3015.4500	dNTP-Set (Na-salt) - 100 mM	20 x 100 µmol	825.00

## IV. PCR Related Products

### Nucleotides

Prod.No.	Product	Pack-size	Price in €
M3439.0000	dTMP	on request	on request
M3400.0100	dTTP Powder, ca. 99.0%	100 mg	106.17
M3400.0250	dTTP Powder, ca. 99.0%	250 mg	200.54
M3400.1000	dTTP Powder, ca. 99.0%	1 g	589.84
M3021.0020	dTTP solution	20 µmol	46.73
M3021.0100	dTTP solution	100 µmol	152.72
M3404.0100	dUTP Powder, min. 96.0%	100 mg	106.17
M3404.0250	dUTP Powder, min. 96.0%	250 mg	200.54
M3404.1000	dUTP Powder, min. 96.0%	1 g	589.84
M3022.0020	dUTP solution	20 µmol	32.44
M3022.0100	dUTP solution	100 µmol	100.27
M3022.5100	dUTP solution	5 x 100 µmol	471.87
M3016.0200	ready to use PCR dNTP-Mix (Na-salt) - 10 mM	200 µL	20.00
M3016.1010	ready to use PCR dNTP-Mix (Na-salt) - 10 mM	1 mL	60.00
M3017.1002	ready to use PCR dNTP-Mix (Na-salt) - 2 mM	1 mL	25.00
M3017.5002	ready to use PCR dNTP-Mix (Na-salt) - 2 mM	5 x 1 mL	70.00

### dNTPs solutions and mixes

Genaxxon bioscience deoxynucleotide triphosphates are supplied as stabilised, time-saving, ready-to-use solutions at a concentration of 100 mM or 50 mM. They can be used directly in DNA sequencing, cDNA synthesis, labelling and PCR technique: deoxynucleotide triphosphate is guaranteed for high purity, free of nuclease activities and rigorously controlled. The Genaxxon bioscience deoxynucleotides are supplied in purified water, available as individual products or in a convenient mix containing dCTP, dGTP and dTTP (or dUTP).

## Polymerase Chain Reaction (PCR)

### How does the Polymerase Chain Reaction (PCR) work?

Double stranded DNA is denatured to single stranded DNA by increased temperature. A short complementary piece of DNA (the primer) could bind to the sequence of the single stranded fragment of DNA (template). The primer recognises the template and binds (anneals) to his recognition sequence. The 3'-end of the primer is used by DNA polymerase to synthesise a new DNA strand (elongation/extension). The three steps of one doubling cycle (denaturation, annealing and elongation) depend on the temperature. So the newly formed double stranded DNA is denatured again at 94-97°C. By lowering the temperature the primers anneal at 35-72°C (the exact temperature depends on the primer, respective its sequence), and the new product is synthesised at 72°C, which is the optimal temperature for *Taq* DNA polymerase.

Each PCR cycle doubles the amount of DNA. After 25 cycles an amount of  $3,2 \times 10^7$  DNA molecules is theoretically amplified. In this way the DNA can be amplified exponentially, resulting in high amounts of double-stranded DNA of the same length and sequence.

### DNA concentration and GC content

DNA preparation should not be impure. The ratio at OD<sub>260/280</sub> should range from 1.8 to 2.0. Impurities, resulting by the plasmid prep and/or DNA purification kits can have strong inhibitory effects on PCR (> 0.5 mM EDTA/EGTA, > 25 mM sodium chloride, > 5 mM sodium acetate, > 1% (v/v) isopropanol or ethanol, > 0.2% (v/v) phenol or > 0.005% (v/w) SDS).

The concentration of DNA template depends on the source (pg up to µg). Normally used concentration are 0.1-1.0 µg for mammalian genomic DNA and 10 - 100 ng for plasmid DNA per 50 µl reaction.

PCR with GC-rich templates (> 60%) are more difficult. This is mainly caused by the formation of stable secondary structures that reduce the efficiency of the polymerase reaction. Good results have been obtained by the addition of glycerol, DMSO (5-20%), formamide (5-20%) or tetramethylammonium chloride (0.01-10 mM) to the reaction mix.

### Primer, Primer design, Melting and annealing temperature

The concentration of each primer should be between 0.1 and 0.8 µM. For most applications 0.2 - 0.4 µM produces satisfactory results.

Too high primer concentrations increase the chance of mispriming, which results in non-specific PCR products. Limiting primer concentrations result in extremely inefficient PCR reactions.

- PCR primers are usually 15-30 nucleotides in length.
- The GC content should be 40-60%.

- The C and G nucleotides should be distributed uniformly within the full length of the primer.
- More than three G or C nucleotides at the 3'-end of the primer should be avoided, as non-specific priming may occur.
- Primer should not be self-complementary (hairpin) or complementary to any other primer in the reaction mixture (primer-dimer).
- The melting temperature of flanking primers should not differ by more than 5°C.
- If primers are degenerate, at least 3 conservative nucleotides must be located at the primer's 3'-end.
- All possible sites of complementarities between primers and the template DNA should be noted.

If the primer is shorter than 25 nucleotides, the approx. melting temperature ( $T_m$ ) is calculated using the following formula:  
 $T_m = 4(G + C) + 2(A + T)$

If the primer is longer than 25 nucleotides, the melting temperature should be calculated using specialised computer software.

The optimal annealing temperature has to be determined experimentally. As a starting point, an annealing temperature 5°C below the estimated melting temperature ( $T_m$ ) can be used.

### MgCl<sub>2</sub> and MgSO<sub>4</sub>

The standard polymerase buffer works well for a wide range of templates and primers but may not be optimal for any particular combination. Especially the concentration of Mg<sup>2+</sup> ions is critical and should be optimised. A series of PCR experiments should be carried out with MgCl<sub>2</sub> for *Taq* DNA polymerase, concentrations varying from 1.5 to 4 mM in 0.5-mM steps and from 1 - 10 mM with MgSO<sub>4</sub> for *Pyrococcus spec.* DNA polymerase. Limiting Mg<sup>2+</sup> ions concentrations result in extremely inefficient PCR reactions. Too high Mg<sup>2+</sup> ions concentrations result in non-specific PCR products.

High concentrations of chelating agents (such as EDTA) and negatively charged ionic groups (such as phosphates) should be avoided. Some PCR reaction buffers include added NH<sub>4</sub><sup>+</sup> ions. It has been shown that the presence of NH<sub>4</sub><sup>+</sup> ions results in a high specificity of the primer-template binding over a broad temperature range.

### dNTPs (2'-deoxynucleotide 5'-triphosphate)

The concentration of each dNTP (dATP, dCTP, dGTP and dTTP) should be 200 µM. Too high concentrations of dNTPs inhibit the PCR reaction

#### *Taq* DNA polymerase from *Thermus aquaticus*

*Taq* DNA polymerase is a thermostable enzyme. The enzyme consists of a single polypeptide with a molecular weight of 94 kDa isolated from eubacterium *Thermus aquaticus*. It has a 5'→3' polymerase activity in the presence of magnesium ions (MgCl<sub>2</sub>) and a 5'→3' exonuclease activity. Effective for PCR products up to 5-10 kb. *Taq* DNA polymerase adds extra A nucleotides to the 3'-ends of PCR products, these fragments can be cloned into T/A vectors.

Error rate (x10<sup>-5</sup>): 2.4

One minute extension time is sufficient for PCR fragments up to 1-2 kb. When larger DNA fragments are amplified, the extending time is usually increased by 1 min for each 1000 bp.

For 50 µl reaction use 0.5 - 2 units.

#### *Pfunds* DNA polymerase from *Pyrococcus furiosus*

*Pfunds* DNA polymerase, isolated from the hyperthermophilic archae bacteria *Pyrococcus furiosus* is a thermostable polymerase of approximately 92 kDa. The enzyme replicates DNA at 75°C, catalysing the polymerisation of nucleotides into duplex DNA in the 5'→3' direction in the presence of magnesium (prefers MgSO<sub>4</sub>). Unlike *Taq* DNA polymerase, *Pfunds* DNA polymerase possesses 3' to 5' exonuclease proofreading activity that enables the polymerase to correct nucleotide misincorporation errors. This means that *Pfunds* DNA polymerase-generated PCR fragments will exhibit the lowest error rate of any thermostable DNA Polymerase, a 12-fold increase in fidelity of DNA synthesis compared with *Taq* DNA polymerase. *Pfunds* DNA polymerase is recommended for use in PCR and primer extension reactions that require high-fidelity synthesis. *Pfunds* DNA polymerase generated PCR fragments are blunt-ended, which can be used directly for blunt end ligation. *Pfunds* DNA Polymerase shows a lower extension rate compared to *Taq* DNA Polymerase (0.5 kb/min), so 2 min extension time is recommended for every 1 kb to be amplified.

*Pfunds* DNA Polymerase prefers MgSO<sub>4</sub> to MgCl<sub>2</sub>.

Error rate (x10<sup>-5</sup>): 0.2

For 50 µl volume use 1.25 - 2.5 units

One minute extension time is sufficient for PCR fragments up to 0.5-1 kb.

### *Taq* DNA Polymerase S

(DNA Polymerase for high specificity PCR)

Prod.No.	Product	Pack-size	Price in €
M3001.0250	Taq Polymerase S (high specificity)	250 units	50.00
M3001.0500	Taq Polymerase S (high specificity)	2 x 250 units	65.00
M3001.1000	Taq Polymerase S (high specificity)	4 x 250 units	120.00
M3001.2500	Taq Polymerase S (high specificity)	10 x 250 units	275.00
M3001.5000	Taq Polymerase S (high specificity)	20 x 250 units	525.00

The Genaxxon bioscience *Taq* Polymerase is a highly processive 5' - 3' DNA polymerase, lacking 3' - 5' exonuclease activity. Amplification of DNA fragments (100 bp to 10 kb) can be achieved with this enzyme. The high fidelity of Genaxxon bioscience *Taq* Polymerase S allows amplification of highly specific DNA fragments up to 10 kb.

## IV. PCR Related Products

### Taq DNA Polymerase E (DNA Polymerase for high efficiency PCR)

Prod.No.	Product	Pack-size	Price in €
M3043.0250	Taq Polymerase E (high efficiency)	250 units	50.00
M3043.0500	Taq Polymerase E (high efficiency)	2 x 250 units	65.00
M3043.1000	Taq Polymerase E (high efficiency)	4 x 250 units	120.00
M3043.2500	Taq Polymerase E (high efficiency)	10 x 250 units	275.00

The Genaxxon bioscience *Taq* Polymerase is a highly processive 5' - 3' DNA polymerase, lacking 3' - 5' exonuclease activity. Amplification of DNA fragments (100 bp to 10 kb) can be achieved with this enzyme. The high processivity of Genaxxon bioscience *Taq* Polymerase allows amplification of DNA fragments up to 10 kb. Genaxxon bioscience *Taq* Polymerase E is delivered with 10X (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> reaction buffer and separate MgCl<sub>2</sub>.

### RedTaq DNA Polymerase (Taq DNA polymerase labelled with a red dye)

Prod.No.	Product	Pack-size	Price in €
M3305.0250	RedTaq (Taq-polymerase labeled with red pigment)	250 units	75.00
M3305.0500	RedTaq (Taq-polymerase labeled with red pigment)	500 units	95.00
M3305.2500	RedTaq (Taq-polymerase labeled with red pigment)	10 x 250 units	375.00

The Genaxxon bioscience RedTaq DNA Polymerase is comparable to M3001 but labelled with a red pigment to enable visualisation of appropriate pipeting and mixing.

### Pwo DNA Polymerase (Proof reading polymerase)

Prod.No.	Product	Pack-size	Price in €
M3002.0100	Pwo Polymerase	100 units	35.00
M3002.0500	Pwo Polymerase	2 x 250 units	155.00
M3002.1250	Pwo Polymerase	5 x 250 units	340.00

Genaxxon bioscience *Pwo* DNA Polymerase is a highly processive 5' - 3' DNA polymerase with additional 3' - 5' exonuclease activity (proof reading). The Genaxxon bioscience *Pwo* DNA Polymerase is the recombinant form, originally isolated from the thermophilic archaeobacterium *Pyrococcus woesei*. The enzyme has no detectable 5' - 3' exonuclease activity. It exhibits increased thermal stability as well as a tenfold increase in fidelity of DNA synthesis compared to *Taq* DNA polymerase. *Pwo* DNA polymerase generates blunt-ended PCR products.

### Pfunds DNA Polymerase (Proof-reading polymerase)

Prod.No.	Product	Pack-size	Price in €
M3004.0250	<i>Pfunds</i> proof reading polymerase	250 units	75.00
M3004.0500	<i>Pfunds</i> proof reading polymerase	2 x 250 units	145.00
M3004.1250	<i>Pfunds</i> proof reading polymerase	5 x 250 units	299.00

The Genaxxon bioscience *Pfunds* DNA Polymerase is a thermostable enzyme possessing 5'-3' DNA polymerase and 3'-5' proof reading exonuclease activities. It is isolated from the hyperthermophilic marine archae *Pyrococcus furiosus* (*Pfu*). The enzyme provides extremely high fidelity. The enzyme is not able to amplify long fragments as efficiently as *Taq* DNA polymerase because of its very high exonuclease activity. *Pfunds* DNA Polymerase generates blunt-ended PCR products.

### ReproFast DNA Polymerase (Proof-reading polymerase)

Prod.No.	Product	Pack-size	Price in €
M3003.0100	ReproFast Polymerase	100 units	42.00
M3003.0250	ReproFast Polymerase	250 units	85.00
M3003.1250	ReproFast Polymerase	5 x 250 units	335.00

ReproFast high-fidelity DNA Polymerase provides high overall performance and more robust amplification of longer targets due to Genaxxon's polymerase-enhancing factor. The ReproFast DNA Polymerase improves the yield of DNA amplicons compared to that from standard *Pfu*- or *Pwo* DNA polymerase. It enhances overall PCR performance, including shorter extension times, higher yield and greater target length capability. The Genaxxon bioscience ReproFast DNA Polymerase is a unique system designed specially to produce a high yield of PCR products from various sources of DNA that can be used to replace *Taq*, *Pfu* or *Pwo* DNA polymerase. The main application is for amplification of long target sequences, including genomic DNA giving high accuracy for cloning experiments. With ReproFast it will be possible to amplify fragments up to 5 kb from human genomic DNA and up to 7 kb

from lambda DNA (Barnes (1994) Proc. Natl. Acad. Sci. USA 91. 2216-2220).

**HotStart Taq DNA Polymerase**  
(with antibody)

Prod.No.	Product	Pack-size	Price in €
M3006.0200	HotStart Taq with Antibody	200 units	70.00
M3006.1000	HotStart Taq with Antibody	1000 units	275.00

The Genaxxon bioscience HotStart Taq Polymerase is the same Taq Polymerase as Cat# M3001, but mixed with special HotStart antibody for HotStart PCR.

**SuperHot Taq DNA Polymerase for qPCR**

(For multiplex PCR. For Realtime PCR, for PCR of low amount of DNA (very low copy number))

Prod.No.	Product	Pack-size	Price in €
M3307.0250	SuperHot Taq-Polymerase for qPCR	250 units	90.00
M3307.1000	SuperHot Taq-Polymerase for qPCR	1000 units	275.00

The Genaxxon bioscience SuperHot Taq DNA Polymerase is a modified form of Taq DNA polymerase, which is activated by heat treatment. A chemical moiety is attached to the enzyme at the active site, which renders the enzyme inactive at room temperature. Thus, during setup and the first PCR cycle, the enzyme is not active and misprimed primers are not extended. As a result specificity and yield are increased compared to standard Taq DNA polymerase. Additionally, difficult targets with high GC-content can be amplified. Sensitivity improves multiplex PCR, an applied PCR technique that amplifies several specific targets simultaneously. Applications that previously required two or more reactions can be performed in a single reaction tube. Hence, multiplexing represents a substantial saving of time and reagents.

**GreenMasterMix (2X)**

(for quantitative PCR with or without fluorescent dye and passive reference dye)

Prod.No.	Product	Pack-size	Price in €
M3023.0100	GreenMasterMix (2X)	2.5 mL	115.00
M3023.0500	GreenMasterMix (2X)	12.5 mL	475.00
M3011.0100	GreenMasterMix (2X) Low ROX	2.5 mL	115.00
M3011.0500	GreenMasterMix (2X) Low ROX	12.5 mL	475.00
M3052.0100	GreenMasterMix (2X) High ROX	2.5 mL	115.00
M3052.0500	GreenMasterMix (2X) High ROX	12.5 mL	475.00

The Genaxxon bioscience GreenMaster PCR Mix (2X) is a ready-to-use PCR mixture containing all reagents required for qPCR (except template and primer). The mix offers dUTP instead of dTTP to prevent carry-over contaminations of DNA from previous PCR reactions. The mastermix contains ROX as passive reference dye and EvaGreen as fluorescent dye. GreenMasterMix is optimized for high specificity and sensitivity because of an optimized Hotstart polymerase and the special formulation of our reaction buffer.

**Data Comparison between different DNA-Polymerases from Genaxxon bioscience**

Product Name	Taq S	Taq E	DF-Taq	PCR Mastermix	RedTaq	PCR RedMaster (2X)	LongMax Kit
Cat #	M3001	M3043	M3185	M3014	M3305	M3029	M3000
5' - 3' polymerase	yes	yes	yes	yes	yes	yes	yes
5' - 3' exonuclease	yes	yes	yes	yes	yes	yes	yes
3' - 5' exonuclease	no	no	no	no	no	no	no
dNTPs incorporation (nucleotides/sec)	35-100	35-100	35-100	35-100	35-100	35-100	35-100
Error rate (x10 <sup>-8</sup> )	3.3	3.8	3.8	3.3	3.8	3.8	3.8
Thermostability and remaining activity at 95 ° C	40 min >50%	40 min >50%	40 min >50%	40 min >50%	40 min >50%	40 min >50%	40 min >50%
Longest amplicons	> 7 kb	10 kb	10 kb	> 7 kb	8kb	8kb	> 12 kb
Addition of poly A	yes	yes	yes	yes	yes	yes	yes
Application	High specificity PCR	High efficiency PCR	High specificity PCR	High specificity PCR	High specificity PCR	High specificity PCR	High efficiency PCR

## IV. PCR Related Products

### Data Comparison between different DNA-Polymerases from Genaxxon bioscience

Product name	HotStart	SuperHot	HotStart Mastermix	Green/ Probe Mastermix (2X)	Pfunds	ReproFast	KOD/ ReproHot
Cat #	M3006	M3307	M3007	M3023/M3011 M3052/M3010	M3004	M3003	M3012
5' - 3' polymerase	yes	yes	yes	yes	yes	yes	yes
5' - 3' exonuclease	yes	yes	yes	yes	yes	yes	yes
3' - 5' exonuclease	no	no	no	no	yes	yes	yes
dNTPs incorporation (nucleotides/sec)	30-60	35-100	35-100	35-100	ca. 10	25-50	30-60
Error rate (x10 <sup>-8</sup> )	-	4.0	4.0	4.0	0.55	0.65	0.65
Thermostability and remaining activity at 95 °C	40 min >50%	60 min >70%	60 min >70%	60 min >70%	90 min >70%	60 min >70%	40 min >50%
Longest amplicons	10kb	8 kb	8 kb	8 kb	5 kb	> 7 kb	> 7 kb
Addition of poly A	yes	yes	yes	yes	blunt end	blunt end	blunt end
Application	Hot Start PCR	Hot Start PCR	High specificity PCR	High specificity	High fidelity PCR	High fidelity PCR Long PCR	High fidelity PCR Hot Start PCR

#### Remarks

M3043 is a Taq-Polymerase optimally suited for colony screens.  
M3185 is a Taq-Polymerase that is tested for contaminants of 16S-DNA.  
M3305 is a Taq-Polymerase that contains a red dye enabling a visualisation of the pipetting procedure.  
M3029 is a PCR-Mastermix containing a red dye. No loading buffer necessary.  
M3006 is a Hotstart DNA-polymerase with a separate Antibody.  
M3307 is a chemically modified Hotstart DNA-polymerase without antibody.  
M3003 is a DNA-polymerase for long fragments with very low error rate.  
M3012 is a Proof-reading DNA-polymerase with antibody.  
M3000 contains a Taq-Polymerase and a special buffer system that enables PCR of very long fragments.  
M3023 is a Hotstart Mastermix containing fluorescent dye  
M3011 is a Hotstart Mastermix containing fluorescent dye and Low ROX as internal standard  
M3052 is a Hotstart Mastermix containing fluorescent dye and High ROX as internal standard  
M3010 is a Hotstart Mastermix containing fluorescent dye and ROX as internal standard for Real-time PCR

## PCR Miscellaneous

### *Tth* Inorganic Pyrophosphatase (thermostable)

Prod.No.	Product	Pack-size	Price in €
M3013.0250	<i>Tth</i> Inorganic Pyrophosphatase Thermostable	250 units	84.00
M3013.1000	<i>Tth</i> Inorganic Pyrophosphatase Thermostable	1000 units	267.75

Native, thermostable *Tth* Inorganic Pyrophosphatase is a hydrolase purified from *Thermus thermophilus*. *Tth* Inorganic Pyrophosphatase catalyses the conversion of inorganic pyrophosphate to orthophosphate in a reaction where pyrophosphate is accumulated. These include DNA synthesis and amplification where *Tth* Inorganic Phosphatase provides enhanced polymerisation, by removing inhibiting amounts of pyrophosphates in the reaction.

PCR is inhibited by the presence of pyrophosphate even at very low concentrations. The introduction of *Tth* Inorganic Pyrophosphatase into the reaction mixture greatly enhances amplification and provides superior results. The addition of 1 unit *Tth* Inorganic Pyrophosphatase to 10 units of DNA Polymerase can double the level of PCR amplification. Moreover *Tth* Inorganic Pyro-phosphatase enables longer DNA fragments to be processed successfully and provides increased fidelity.

### Mineral Oil in a dropper bottle

Prod.No.	Product	Pack-size	Price in €
M3024.0810	Mineral Oil	8 x 10 mL	48.12

#### Specifications

Provided in dropper bottles; boiling range (1000 hPa): 300-400 °C; density (d<sub>20</sub> °C): 0.818-0.875 dyn; viscosity: min. 25-80 mPa\*s (20 °C); DNase and RNase activities not detected.

#### Storage and shipment

shipped and stored at -20 °C

#### Usage

One drop of mineral oil loaded on top of the PCR reaction mixture avoids evaporation at high temperature.

## Error rate of different DNA Polymerases

	Error rate (x10 <sup>-6</sup> )	Accuracy (b)	Clones with mutation (%) (10 <sup>6</sup> -time Amplification, 1 kb)
<i>Pfu</i> Polymerase	0.55	1 818 000	1.1%
ReproFast Polymerase	0.65	1 538 000	1.3%
<i>Taq</i> Polymerase S	3.3	303 000	6.6%
<i>Pfu</i> Ultra™ Polymerase	0.43	2 326 000	0.9%
<i>Pfu</i> , <i>Pfu</i> Turbo® Polymerase	1.3	769 000	2.6%
DeepVent® Polymerase	2.7	370 000	5.4%
Vent-R® Polymerase	2.8	357 000	5.6%
Platinum®, <i>Pfx</i> Polymerase	3.5	286 000	7.0%

Fehlerraten wurden im empfohlenen PCR Puffer und bei Standard PCR-Bedingungen ermittelt.  
Daten für DNA Polymerasen anderer Hersteller wurden von Stratagene übernommen.  
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## HotStart & Realtime PCR

### Real-Time PCR - The Theory

Theoretically, there is a quantitative relationship between the amount of starting material and amount of PCR product at any given cycle. In practice, though, there is often no direct correlation. The development of real-time quantitative PCR has eliminated the variability traditionally associated with quantitative PCR, thus allowing the routine and reliable quantification of PCR products. This method, therefore, now provides investigators with the ability to perform very sensitive, accurate, and reproducible measurements of levels of gene expression. In addition, quantitative PCR can be used in other applications such as measuring viral load, performing allelic discrimination studies, or optimising PCR conditions.

### Real-Time PCR Chemistry

Real-time systems for PCR were improved by probe-based, rather than intercalator-based, PCR product detection. The principal drawback of intercalator-based detection of PCR product accumulation is that both specific and non-specific products generate a signal. The development of fluorogenic probes made it possible to eliminate post-PCR processing for the analysis of probe degradation. The probe is a dual labelled oligonucleotide with both a reporter fluorescent and a quencher dye attached. While the probe is intact, the proximity of the quencher almost completely reduces the fluorescence by Förster resonance energy transfer (FRET) through space.

If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of *Taq* DNA polymerase during the extension phase. Cleavage of the probe separates the reporter dye from quencher dye, increasing the reporter dye signal. Cleavage also removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase of fluorescence intensity proportional to the amount of amplicon produced.

### Real-Time PCR Quantification

The ability to monitor the real-time progress of the PCR completely revolutionised the way of PCR-based quantification of DNA and RNA. Reactions are characterised by the point in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase (above base line) in fluorescence is observed. In the initial cycles of PCR, there is only little change in fluorescence signal. This defines the baseline for the amplification plot. A fixed threshold should be set above this baseline. An increase in fluorescence above the threshold indicates the detection of accumulated PCR product. The parameter "CT" (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. A plot of the log of initial target copy number for a set of standards versus CT is a straight line. Quantification of the amount of target in unknown samples is accomplished by measuring CT and using the standard curve to determine starting copy number.

### Primer and Probe Design

Primers and probes should be carefully designed. Important parameters include a  $T_m$  for the probe that is approximately 10°C higher than the primers ensuring full hybridisation while primer extension.

Small amplicons are favoured because they promote high-efficiency assays that work the first time. In addition, high-efficiency assays enable relative quantification to be performed using the comparative CT method (DDCT). This method increases sample throughput by eliminating the need for standard curves when looking at expression levels of a target relative to a reference control.

GC-rich sequences are susceptible to non-specific interactions that may reduce reaction efficiency and produce non-specific signal in SYBR Green assays. For the same reason, primer and probe sequences containing runs of four or more G bases should be avoided. A/T-rich sequences require longer primer and probe sequences in order to obtain the recommended  $T_m$ . This is rarely a problem for quantitative assays; however, probes approaching 40 base pairs can exhibit less efficient quenching and produce lower synthesis yields.

## IV. PCR Related Products

### Useful Hints

#### 1. Preparation, Quantification, and Determination of the quality of DNA and RNA

##### 1a. Template preparation and quality

Since PCR consists of multiple rounds of enzymatic reactions, it is more sensitive to impurities such as proteins, phenol/chloroform, salts, EDTA, and other chemical solvents than single-step enzyme-catalysed reactions. Purity of nucleic acid templates is particularly important for real-time PCR, since contaminations can interfere with fluorescence detection.

##### 1b. Determining concentration and purity of nucleic acid

The concentration of DNA and RNA should be determined by measuring the absorbance at 260 nm ( $A_{260}$ ) in a spectrophotometer. For accuracy, absorbance reading at 260 nm should fall between 0.15 and 1.0. Brief guides to spectrophotometric and molar conversion values for different nucleic acid templates are listed in Table 1 and 2.

Table 1: Spectrophotometric conversions for nucleic acid templates

1 $A_{260}$ unit *	Concentration ( $\mu\text{g/mL}$ )
Double-stranded DNA	50
Single-stranded DNA	33
Single-stranded RNA	40
* Absorbance at 260 nm = 1 (1 cm detection path length)	

Table 2: Molar conversions for nucleic acid templates

Nucleic acid	Size	pmol/ $\mu\text{g}$	Molecules/ $\mu\text{g}$
1kb DNA	1000 bp	1.52	$9.1 \times 10^{11}$
pUC19 DNA	2686 bp	0.57	$3.4 \times 10^{11}$
pTZ18R DNA	2870 bp	0.54	$3.2 \times 10^{11}$
pBluescript II DNA	2961 bp	0.52	$3.1 \times 10^{11}$
Lambda DNA	48,502 bp	0.03	$1.8 \times 10^{10}$
Typical mRNA	1930 nt	1.67	$1.0 \times 10^{12}$
<b>Genomic DNA</b>			
<i>Escherichia coli</i>	$4.7 \times 10^6$	$3.0 \times 10^{-4}$	$1.8 \times 10^8$ **
<i>Drosophila melanogaster</i>	$1.4 \times 10^8$ *	$1.1 \times 10^{-5}$	$6.6 \times 10^5$ **
<i>Mus musculus</i> (mouse)	$2.7 \times 10^9$ *	$5.7 \times 10^{-7}$	$3.4 \times 10^5$ **
<i>Homo sapiens</i> (human)	$3.3 \times 10^9$ *	$4.7 \times 10^{-7}$	$2.8 \times 10^5$ **

\* Base pairs in haploid genome

\*\* For single-copy genes

**NOTE:** Absorbance measurements cannot discriminate between DNA and RNA. Depending on the method used for template preparation, DNA may be contaminated with RNA, or RNA may be contaminated with DNA, and either of these will result in misleading high  $A_{260}$  values. It is particularly important to bear this in mind when preparing standards for absolute quantification.

The ratio between the absorbance values at 260 nm and 280 nm gives an estimate of the purity of DNA or RNA. To determine nucleic acid purity, we recommend measuring absorbance in 10 mM Tris-HCl, pH 7.5. Pure DNA and RNA have  $A_{260}/A_{280}$  ratios of 1.8 - 2.0 and 1.9 - 2.1 (2.3 \*) respectively. Lower ratios indicate the presence of contaminants such as proteins.

\* Values up to 2.3 are routinely obtained for pure RNA with some spectrophotometers.

#### 2. Storage of DNA and RNA

Purified RNA should be stored at  $-20^\circ\text{C}$  or  $-70^\circ\text{C}$ , in RNase-free water.

Purified DNA should be stored at  $-20^\circ\text{C}$  or  $-70^\circ\text{C}$  under slightly basic conditions (e.g. Tris-HCl, pH 8.0) because acidic conditions can cause hydrolysis of DNA. Diluted solutions of nucleic acids (e.g. dilution series used as standards) should be stored in aliquots and thawed once only. We recommend storage of aliquots in siliconised tubes where possible. This avoids adsorption of the nucleic acid to the tube walls, which would reduce the concentration of nucleic acid in solution.

#### 3. Primer Design, Concentration, and Storage

Prerequisites for successful PCR include design of optimal primer pairs, use of appropriate primer concentrations, and correct storage of primer solutions. Guidelines are provided in Table 3. Since fluorescence from SybrGreen increases strongly upon binding of the dye to any double-stranded DNA, it is particularly important to minimise non-specific primer annealing by careful primer design.

Table 3: General guidelines for PCR primers

Length	18 - 30 nucleotides.																				
GC-content	40% - 60%																				
$T_M$	<p>There are many formula to calculate <math>T_M</math> temperatures. A simplified formula for estimating melting temperature:  <math>T_M = 2^\circ\text{C} \times (\text{number of [A+T]}) + 4^\circ\text{C} \times (\text{number of [G+C]})</math>  Whenever possible, design primer pairs with similar <math>T_M</math> values.</p> <p>Optimal annealing temperatures may be above or below the estimated <math>T_M</math>. As a starting point, use an annealing temperature <math>5^\circ\text{C}</math> below <math>T_M</math>.</p> <p>Primer <math>T_M</math>s between <math>58^\circ\text{C}</math> and <math>60^\circ\text{C}</math> enables the use of universal thermal cycling parameter.</p>																				
Sequence	<p>Ideally, the length of the PCR product is 100 - 250 bp.</p> <p>Avoid complementarity of 2 or more bases at the 3' ends of primer pairs to reduce primer-dimer formation.</p> <p>Avoid mismatches between the 3' end of the primer and the target-template sequence.</p> <p>Avoid runs of 3 or more Gs or Cs at the 3' end.</p> <p>A 5'-G has to be avoided as 5'-Gs quench the fluorescence signal.</p> <p>Avoid a 3' end T. Primers with a T at the 3' end have a greater tolerance to mismatch.</p> <p>Avoid complementary sequences within a primer sequence and between the primer pair.</p> <p>Commercially available computer software (e.g. OLIGO 6, Rychlik, 1999) or web-based tools (e.g. Primer3, Steve Rosen&amp;Helen Skaletsky, 2000 (<a href="http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi">http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi</a>)) can be used for primer design. Use the software to minimise the likelihood of formation of stable primer-dimers.</p>																				
Special considerations for design of RT-PCR primers	<p>Design primers so that one half of the primer hybridises to the 3' end of one exon and the other half to the 5' end of the adjacent exon. Primers will anneal to cDNA synthesised from spliced mRNAs, but not to genomic DNA. Thus, amplification of contaminating DNA is eliminated.</p> <p>Alternatively, RT-PCR primers should be designed to flank a region that contains at least one intron. Products amplified from cDNA (no introns) will be smaller than those amplified from genomic DNA (containing introns). Size difference in products allows detection of contaminating DNA by melting curve analysis. If genomic DNA is detected, first treat the template RNA with RNase-free DNase, or if this is not possible, redesign primers to avoid amplification of genomic DNA</p>																				
Concentration	<p>Spectrophotometric conversion for primers:  1 <math>A_{260}</math> unit = 20 - 30 <math>\mu\text{g}/\text{mL}</math></p> <p>Molar conversions:</p> <table border="1"> <thead> <tr> <th>Primer length</th> <th>pmol/<math>\mu\text{g}</math></th> <th>10 pmol *</th> <th>15 pmol **</th> </tr> </thead> <tbody> <tr> <td>18mer</td> <td>168</td> <td>59 ng</td> <td>89 ng</td> </tr> <tr> <td>20mer</td> <td>152</td> <td>66 ng</td> <td>99 ng</td> </tr> <tr> <td>25mer</td> <td>121</td> <td>83 ng</td> <td>124 ng</td> </tr> <tr> <td>30mer</td> <td>101</td> <td>99 ng</td> <td>149 ng</td> </tr> </tbody> </table> <p>* Final concentration: 0.5 <math>\mu\text{M}</math> in 20 <math>\mu\text{L}</math>.  ** Final concentration: 0.3 <math>\mu\text{M}</math> in 50 <math>\mu\text{L}</math>.</p> <p>Depending on the real-time cyler used, optimal primer concentrations may vary as indicated in the protocols</p>	Primer length	pmol/ $\mu\text{g}$	10 pmol *	15 pmol **	18mer	168	59 ng	89 ng	20mer	152	66 ng	99 ng	25mer	121	83 ng	124 ng	30mer	101	99 ng	149 ng
Primer length	pmol/ $\mu\text{g}$	10 pmol *	15 pmol **																		
18mer	168	59 ng	89 ng																		
20mer	152	66 ng	99 ng																		
25mer	121	83 ng	124 ng																		
30mer	101	99 ng	149 ng																		
Storage	<p>Lyophilised primers can be stored for at least 2 years at <math>+4^\circ\text{C}</math>.</p> <p>It is possible to dissolve lyophilised primers in a small volume of TE buffer to make a concentrated stock solution. Prepare small aliquots of working solutions containing 10 pmol/<math>\mu\text{L}</math> to avoid repeated thaw- freeze cycles. Store all primer solutions at <math>-20^\circ\text{C}</math>. Primer quality can be checked on a denaturing polyacrylamide gel (a single band should be seen).</p>																				

## IV. PCR Related Products

### Troubleshooting

Problem	Comments - Suggestions
<b>No PCR product / Product detected very late in PCR / Only primer-dimers detected.</b>	
Annealing time too short	Increase annealing time by at least 10 seconds. Annealing times in Block Thermal Cyclers: > 20 seconds Annealing times in Capillary Thermal Cyclers: > 7 seconds Too short annealing times are often seen together with LightCycler capillaries made from polycarbonate. Annealing time should be at least 10 seconds.
Extension time too short	Always use extension times of 1 minute per 1 kb (30 seconds up to 500 bp). For templates longer than 5 kb it might be necessary to increase extension time by additional 10 seconds per 1 kb.
Denaturation time too short	High GC content regions may not denature well during thermal cycling, leading to a less efficient reaction. Increase denaturation time and/or denaturation temperature.
MgCl <sub>2</sub> concentration not optimal	Always start with the MgCl <sub>2</sub> concentration provided in the Genaxxon bioscience kits and mastermixes. For few targets only, an increase up to 5 mM MgCl <sub>2</sub> may be helpful. Perform titration in 0.5 mM steps.
Pipetting error / missing reagent	Check concentration and storage conditions of reagents, including primers and templates. Repeat PCR.
HotStart <i>Taq</i> not activated	In contrast to antibody inactivated <i>Taq</i> DNA Polymerase the chemically modified <i>Taq</i> DNA Polymerase needs at least 10 minutes at 95 °C to be activated. Check your PCR protocol.
Problem	Comments - Suggestions
<b>No PCR product / Product detected very late in PCR / Only primer-dimers detected.</b>	
PCR product too long	For optimal results, PCR products should be between 100 and 250 bp and should not exceed 500 bp.
PCR annealing temperature too high	Decrease annealing temperature in 3 °C steps. It is very convenient to use a touch-down or gradient programme.
Primer design not optimal	Check for presence of PCR products by melting curve or gel electrophoresis analysis. If no specific PCR products are detected, review the primer design guidelines.
Primer concentration not optimal	Use optimal primer concentrations. In Block Cycler: 0.3 µM each primer In LightCycler: 0.5 µM each primer
Problems with starting template	Check concentrations, storage conditions, and quality of the starting template. If necessary, make new serial dilutions of template nucleic acid from stock solutions. Repeat the PCR using the new dilutions.
Insufficient starting template	Increase the template amount, if possible.
Insufficient number of cycles	Increase cycle numbers
No detection activated	Check that fluorescence detection was activated in the cycling program.
Wrong detection step	Ensure that fluorescence detection takes place during the extension step of the PCR program.
UNG treatment combined with low annealing temperature	If annealing temperatures below 55 °C are necessary for successful PCR, the optimal UNG treatment should be performed using heat-labile UNG only.
Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.
<b>RT-PCR only</b>	
Volumes of RT reaction added were too high	High volumes of RT reaction added to PCR may reduce amplification efficiency. Generally the volume of reverse transcriptase reaction added should not exceed 10% of the final PCR volume.
<b>Fluorescent dye</b>	
Wrong fluorescent dye	Many Real-time PCR machines have detection channels with fixed emission and absorbance wave length. The fixed wave length may not fit with the absorbance and emission spectrum of the used fluorescent dye (example: ROX in LightCycler machines versions 1.2/1.5/2.0).
<b>For ABI Sequence Detection Systems and iCycler only</b>	
Wrong dye layer / filter chosen	Ensure that "SybrGreen" layer/filter is activated.
<b>For LightCycler only</b>	
Chosen fluorescence gains are too low	When using software versions earlier than 3.5, ensure fluorescence gain for channel 1 is set to "15".
<b>Primer-dimers and / or non-specific PCR products</b>	
MgCl <sub>2</sub> concentration not	Always start with MgCl <sub>2</sub> concentration provided in all Genaxxon bioscience Kits, Sets and Mastermixes. For a few targets, an increase in the MgCl <sub>2</sub> concentration may be helpful. Perform

## IV. PCR Related Products

optimal	titration in 0.5 mM steps.
PCR annealing temperature too low	Increase annealing temperature in increments of 2 °C. Most convenient to use a gradient program.
Primer design not optimal	Review primer design. If redesigning of primers is not possible, include additional data acquisition step above $T_m$ of primer-dimers.
PCR product too long	For optimal results, PCR products should be between 100 and 250 bp and should not exceed 500 bp.
Primer-dimers co-amplified	Include an additional data acquisition step in the cycling program as indicated in the protocols to avoid the detection of primer-dimers.
Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.
<b>RT-PCR only</b>	
Contamination with genomic DNA	Pretreat starting RNA template with DNase I. Alternatively, use primers located at splice junctions of the target mRNA to avoid amplification from genomic DNA.
<b>Optional data acquisition step only</b>	
Detection temperature too high	Ensure that the detection temperature is at least 3 °C lower than the $T_m$ of the specific product. When establishing a new primer-template system, always perform a 3-step cycling reaction first, without the optional data acquisition step.

Problem	Comments - Suggestions
<b>No linearity in ratio of CT value / crossing point to log of the template amount</b>	
Template amount too high	Do not exceed maximum recommended amounts of template. For genomic DNA in Block Cyclers: Do not use more than 500 ng template. For genomic DNA in the LightCycler: Do not use more than 1 µg template.
Template amount too low	Increase template amount, if possible.
Primer-dimers co-amplified	Include an additional data acquisition step in the cycling program as indicated in the protocols to avoid the detection of primer-dimers.
<b>RT-PCR only</b>	
Volumes of RT reaction added were too high	High volumes of RT reaction added to PCR may reduce amplification efficiency. Generally the volume of reverse transcriptase reaction added should not exceed 10% of the final PCR volume.
<b>High fluorescence in "No Template" control</b>	
Contamination of reagents	Discard reaction components and repeat with new reagents.
Contamination during reaction steps	Take appropriate safety precautions (e.g. use filter tips). Use uracil-N-glycosylase to prevent carryover from previous reactions.
<b>High fluorescence in "No RT" control reactions (RT-PCR only)</b>	
Contaminating genomic DNA in RNA preparation	Design exon-spanning primers and /or probes to amplify/detect only the cDNA target.
<b>Varying fluorescence intensity</b>	
Real-time cycler contaminated	Decontaminate the real-time cycler according to the supplier's instructions.
Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the supplier's instructions.
<b>ABI Sequence Detection Systems and iCycler only</b>	
Wavy curve at high template amounts	Reduce number of cycles used for baseline calculation.