Ampliqon

RealQ RT-PCR Master Mix Two Step (7mM $MgCl_2$)

(Final $MgCl_2$ is 3.5mM)

With-out Green DNA Dye I

For 200 Reactions of 50µl pr Reactions

Cat. No.: 252407

ROX dye already included in the 2x RealQ RT-PCR Master Mix. No need to add ROX dye.

Cat. No.	Size Reactions	Kit
252403	200	RealQ RT-PCR Master Mix Two Step (3mM $MgCl_2$)
252406	200	RealQ RT-PCR Master Mix Two Step (6mM $MgCl_2$)
252407	200	RealQ RT-PCR Master Mix Two Step (7mM $MgCl_2$)
252410	200	RealQ RT-PCR Master Mix Two Step (10mM $MgCl_2$)
252503	200	RealQ RT-PCR Master Mix Two Step (3mM $MgCl_2$) *with Green DNA dye in mix*
252506	200	RealQ RT-PCR Master Mix Two Step (6mM $MgCl_2$) *with Green DNA dye in mix*
252507	200	RealQ RT-PCR Master Mix Two Step (7mM $MgCl_2$) *with Green DNA dye in mix*
252510	200	RealQ RT-PCR Master Mix Two Step (10mM $MgCl_2$) *with Green DNA dye in mix*
252603	200	RealQ RT-PCR Master Mix Two Step (3mM $MgCl_2$) *with Green DNA dye separate*
252606	200	RealQ RT-PCR Master Mix Two Step (6mM $MgCl_2$) *with Green DNA dye separate*
252607	200	RealQ RT-PCR Master Mix Two Step (7mM $MgCl_2$) *with Green DNA dye separate*
252610	200	RealQ RT-PCR Master Mix Two Step (10mM $MgCl_2$) *with Green DNA dye separate*

Store at -20°C. *Reagent for in-vitro laboratory use only*

Important information for the user

This kit is intended for more experience users that needs high quality products to an affordable price. It is not the intention of this instruction insert to give a complete overview of the Quantitative PCR methods but simply a short guide describing the most important issues for running Quantitative PCR using RealQ-PCR products. For more detailed description please consult the original manuals coming with the Quantitative PCR Instrument.

This protocol describes how to perform Real-Time Reverse Transcription PCR in a two-step procedure. In the two-step procedure, the Reverse Transcription is a separate step from the Real-Time PCR step.

Components already included in the 2x RealQ-PCR Master Mix: Optimized buffer system, ROX Reference dye, dATP, dCTP, dGTP and dTTP.
The $MgCl_2$ concentration is 7mM, which gives an $MgCl_2$ concentration of 3.5mM in the final reaction.

Introduction

Quantitative PCR has become an important tool for SNP and gene expression analysis. Several different fluorescent chemistries exist for either detection of SNP or quantitative gene transcripts. The use of fluorescent probe technologies reduces the risk of sample contamination while maintaining convenience, speed and high throughput screening capabilities. Ampliqon has developed the RealQ-PCR Master Mix, a single-tube 2X reagent ideal for most Quantitative PCR applications. The RealQ-PCR kit support quantitative amplification and detection with multiplex capability. The RealQ-PCR kit has been designed for optimal performance on ABI PRISM™ Instruments, the LightCycler™ Instrument, the Mx4000™ Instrument and the DNA Engine Opticon™ System. The RealQ-PCR kits includes the components necessary for performing PCR amplification, and have been successfully used to amplify and detect a variety of DNA targets such as genomic DNA, cDNA and plasmid DNA.
The RealQ-PCR master mix includes the TEMPase Hot Start DNA polymerase, a modified Taq DNA polymerase with hot start capabilities. The TEMPase Hot Start enzyme improves the PCR amplification reaction by decreasing background from non-specific amplification and increases amplification of desired products.

Materials provided for 200 Real Time PCR Two Step reactions (50 µl pr reaction)

Materials provided (per kit)	Quantity
Reverse Transcriptase Enzyme (200 U/µl)	100 µl
Reverse Transcriptase Buffer (5X)	1 ml
dNTP Mix (10 mM)	800 µl
2x RealQ PCR Master Mix (7mM $MgCl_2$)	4 x 1.25 ml
Glass blocking agents (50X) (LightCycler™)	200 µl
$MgCl_2$ Concentration 25 mM	1.5 ml

Storage Conditions
Upon receipt, store all components at –20°C.
Store the 2X master mix at + 4°C after thawing. Once thawed, full activity is guaranteed for 3 month. Glass blocking agents and $MgCl_2$ can be stored at both -20°C and +4°C.

RNA Isolation

High-quality intact RNA is essential for successful synthesis of full-length cDNA. Isolation of polyA RNA is typically not necessary, although including this step may improve the yield of specific cDNA templates. RNA samples with an $OD_{260/280}$ of 1.8-2.0 are optimal.

cDNA Synthesis Primers

Use of poly(dT)$_{16-25}$ primer is recommended for subsequent amplification of multiple transcripts from a single first-strand synthesis reaction. Random hexamer are efficient primers for detection of multiple short RT-PCR targets. Gene-specific primers anneal only to defined sequences and are use to synthesize cDNA from particular mRNA transcripts rather than from the entire mRNA population in the sample. Using gene-specific primers the specificity of priming can be improved by optimising the annealing and reactions temperature.

1. Strand Synthesis for PCR amplification

One microliter of the cDNA synthesis reaction is a sufficient quantity for efficient amplification of most targets. Excess salt in the cDNA synthesis reaction will inhibit the DNA polymerase in the amplification step. **Note:** To avoid excess salt in the PCR amplification, it can be helpful to include a precipitation step after the cDNA synthesis step, ex. ethanol precipitation or QIAquick PCR purification Kit (Qiagen) can be used.

Protocol First Strand synthesis

Make specific cDNA transcripts, using gene-specific or a pool of cDNA transcripts using random hexamer or poly(dT)$_{16-25}$ primers, before doing Real-Time PCR.

1. In a sterile RNase-free microcentrifuge tube add;

2. Template RNA:

10ng-5µg	Total RNA or
1ng-0.5µg	mRNA or
0.01-0.5µg	Specific RNA

Primer:

0.5µg	Oligo(dT)$_{18}$
0.2µg	Random Hexamer
15-20 pmol	Sequence specific

DEPC-treated water to 11µl

3. Heat the tube to 70°C for 5 minutes to melt secondary structure within the template. Cool the tube immediately on ice to prevent secondary structure from reforming, and then spin briefly to collect the solution at the bottom of the tube.
4. Add the following components in the order shown.

Component	Amount	Final
5X Reverse Transcriptase Buffer	4 µL	1X
dNTP Mix (10 mM)	2.0 µL	1mM
Optional RNA Safe	20 Units	1U/µl
DEPC-treated water to 11µl		

5. Incubate at 37°C for 5 min. If random primer is used incubate at 25°C for 5 min.
6. Add 200 Units Reverse Transcriptase Enzyme. Incubate Reaction Mixture containing;

Oligo(dT)$_{18}$	at 42°C for 60 min
Sequence specific	at 42°C for 60 min
Random Hexamer	at 25°C for 10min and at 42°C for 60 min

7. Stop the reaction by heating to 70°C for 15min. Chill on ice.

8. Important: End the incubation at 95°C for 3 min. to inactive the RT-Enzyme prior to PCR. Inactivation of the RT Enzyme will increase yields of the amplification products.

Place the completed cDNA synthesis on ice or store at -20°C. Use not more than 1µl of the cDNA synthesis for PCR amplification or include a precipitation/column purification step to avoid excess salt in the amplification reaction.

PRE-PROTOCOL CONSIDERATIONS

PCR Primers

It is important especially in Green DNA dye I based Real Time PCR applications to minimize the formation of non-specific amplification products. Especially at low target concentration it is important to use the lowest primer concentration without compromising the efficiency of PCR. The optimal concentration of primer pairs is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration, with minimal or no formation of primer-dimer.
The optimal concentrations of upstream and downstream primers are not always of equal molarity.

Primer concentration for TaqMan probes	Primer concentration for Molecular Beacons	Primer concentration in Multiplex PCR
50 to 600 nM	200 to 600 nM	20 to 200 nM

Primer concentration optimization scheme

PCR probes

The optimal concentration of the experimental probe should be determined empirically. The optimal concentration is the lowest concentration that result in the lowest Ct and an adequate fluorescence for a given target concentration.

TaqMan probes, conc.	Molecular Beacons, conc.
Between 100 to 500 nM in increments of 100 nM	Between 200 to 500 nM in increments of 100 nM.

Probe concentration optimization scheme

Reference Dye

A passive reference dye is included in the 2x RealQ-PCR Master Mix kit to compensate for non-PCR related variations in the fluorescence. The fluorescence from the passive reference dye does not change during the course of the PCR reaction but provide a stable baseline to which samples are normalized. The excitation and emission of the reference dye are 584 nm and 612 nm, respectively.

Magnesium Chloride

The optimal $MgCl_2$ concentration gives maximal amplification of a specific target amplicon with minimal non-specific products and primer-dimer formation. It is important especially in Green DNA I dye based Quantitative applications to optimize the $MgCl_2$ level, to avoid detection of non-specific dsDNA including primer-dimers. In general the $MgCl_2$ concentration in Green DNA I dye based application should be between 1.5 and 5.0 mM. The master mix is supplied with a final $MgCl_2$ concentration of 3.5 mM. For adding extra $MgCl_2$ please consult the below table.

Final $MgCl_2$ conc. in reaction (mM)	3.5	4.0	4.5	5.0
Additional volume of 25 mM $MgCl_2$ per 50 µl reaction (µL):	0	1	2	3

$MgCl_2$ dilution scheme

Preventing Template Cross-Contamination

Due to the high sensitivity of Quantitative PCR it is a risk that reaction may be contaminated with the products of previous runs. To minimize this risk, tubes or plates containing reaction products should not be opened or analyzed by gel electrophoresis in the same laboratory area used to set up reactions.

Glass blocking agents (LightCycler™)

One extra challenge using the LightCycler™ instrument is that the PCR reagents can form precipitate on the glass capillary surface as the Real time PCR progresses. To prevent this event Ampliqon has designed a special reagent for blocking the glass capillaries during the Real Time PCR reaction. The Glass blocking agent comes as a 50X solution (1 µl pr 50 µl PCR reaction).

Protocol

Prior to the experiment, it is prudent to carefully optimize experiment conditions and to include controls at every stage. See pre-protocol considerations for details.

Thaw the 2x RealQ-PCR Master Mix and store on ice. Following initial thawing of the master mix, store the unused portion at +4°C.

Note: Multiple freeze-thaw cycles should be avoided. The Green DNA I dye present in the master mix is light sensitive. Solution containing the Green DNA I dye should be protected from light whenever possible.

Prepare the experimental reaction by adding the components in the following order:

25 µl of 2X master mix
x µl of experimental probe (optimized concentration)
x µl of upstream primer (optimized concentration)
x µl of downstream primer (optimized concentration)

Gently mix the reactions without creating bubbles (do not vortex).

Add x µl of experimental gDNA, cDNA or plasmid DNA to each experimental reaction.

Add Nuclease-free PCR-grade H_2O to adjust the final volume to 50µl (including experimental DNA)

Gently mix the reaction without creating bubbles (do not vortex).

Note: Bubbles interfere with fluorescence detection.

Place the reaction in the instrument and run the appropriate program below.

2-step PCR Program

Cycles	Duration of cycle	Temperature
1[a]	2 minutes	50 °C
1[b]	15 minutes	95 °C
40	15-30 seconds[c]	95 °C
	1.0 minute[d]	55-60 °C[e]

[a] Can be excluded if UNG is not used.
[b] For activation of the TEMPase hot start enzyme.
[c] Varying between thermocycles, used 30 seconds for the ABI PRISM 7700 instrument.
[d] Set the temperature cycler to detect and report fluorescence during the annealing/extension step of each cycle.
[e] Choose an appropriate annealing temperature for the primer set used.

3-step PCR Program

Cycles	Duration of cycle	Temperature
1[a]	2 minutes	50 °C
1[b]	15 minutes	95 °C
40	30 seconds[c]	95 °C
	1.0 minute[d]	55-60 °C[e]
	30 seconds	72 °C

[a] Can be excluded if UNG is not used.
[b] For activation of the TEMPase hot start enzyme.
[c] Varying between thermocycles, used 30 seconds for the ABI PRISM 7700 instrument.
[d] Set the temperature cycler to detect and report fluorescence during the annealing/extension step of each cycle.
[e] Choose an appropriate annealing temperature for the primer set used.

Related Products

Description	Cat. No.
Taq DNA Polymerase (500 Units) *with 10X Ammonium Reaction Buffer* *with 10X Standard Reaction Buffer*	**110303**
Taq DNA Polymerase (500 Units) *with 10X Combination Buffer*	**110403**
Taq DNA Polymerase (500 Units) *with 10X Mg^{++} Free Ammonium Buffer*	**110503**
Taq DNA Polymerase 2.0X Master Mix (100 Reac) *with 2.0 mM MgCl2*	**150301**
Taq DNA Polymerase 2,0X MaMi RED (100 Reac) *with 1.5 mM MgCl2,*	**180301**
Taq DNA Polymerase 2.0X MaMi RED (100 Reac) *with 2.0 mM MgCl2*	**190301**
AccuPOL DNA Polymerase (500 Units)	**210303**
TEMPase Hot Start DNA Polymerase (500Units) *with 10X TEMPase Buffer I* *with 10X TEMPase Buffer II*	**220303**
UniPOL –Long Range PCR (100 Reac)	**270701**
Rapid Ligation Kit (50 React)	**750300**
RT-PCR One Tube (100 Reac)	**740301**
TEMPase Hot Start 2X Master Mix *with TEMPase Buffer I* (100 Reac)	**230301**
TEMPase Hot Start 2X Master Mix *with TEMPase Buffer II* (100 Reac)	**230701**
dNTP Mix (2 x 500µl) (12.5 mM of each dA, dC, dG and dT)	**501004**
dNTP Mix, (2 x 500 µl) (10 mM of each dA, dC, dG and dT),	**502004**
GC5 Value Efficiency, 10^8 Cfu/µg pUC19 Chemically Competent Cells, (10x 200µl)	**812010**
GC5 High Efficiency, 10^9 Cfu/µg pUC19 Chemically Competent Cells, (10x 50µl)	**805010**
GC5 High Efficiency, 10^9 Cfu/µg pUC19 Chemically Competent Cells, (5x 200µl)	**802005**
SuperPath GC10, 10^{10} Cfu/µg pUC19 ElectroCompetent Cells, (5x 80µl)	**830805**
SOC Medium, 10x 10mL	**800000**

NOTICE

In certain countries, patents cover the PCR process. This product is intended for researchers having a license to perform PCR or those not required to obtain a license.