Thermo
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PRODUCT INFORMATION

Thermo Scientific
Maxima SYBR Green qPCR Master Mix (2X), ROX Solution provided

#K0252
For 1000 reactions of 25 µl
Lot _____
Exp. __.____
Store at -20°C in the dark.

www.thermoscientific.com/fermentas

CERTIFICATE OF ANALYSIS

The absence of endo-, exodeoxyribonucleases and ribonucleases confirmed by appropriate quality tests.
Functionally tested in real-time PCR in parallel 25 µl reactions containing 10-fold dilutions of human genomic DNA to demonstrate linear resolution over five orders of dynamic range.

Quality authorized by: Jurgita Zilinskiene

CONTENTS page

COMPONENTS

Component	#K0251 for 200 rxns of 25 µl	#K0252 for 1000 rxns of 25 µl	#K0253 for 4000 rxns of 25 µl
Maxima SYBR Green qPCR Master Mix (2X), no ROX	2x1.25 ml	10x1.25 ml	4x12.5 ml
ROX Solution, 50 µM	50 µl	250 µl	1 ml
Water, nuclease-free	2x1.25 ml	10x1.25 ml	2x30 ml

STORAGE

Store at -20°C in the dark for long term storage or at 4°C for up to one month. Avoid multiple freeze-thawing of ROX Solution.

DESCRIPTION

Thermo Scientific Maxima SYBR Green qPCR Master Mix (2X) is an universal ready-to-use solution for quantitative real-time PCR and two-step real-time RT-PCR on most real-time PCR machines. The master mix includes Maxima® Hot Start *Taq* DNA polymerase and dNTPs in an optimized PCR buffer. It contains SYBR® Green I dye. ROX Solution is provided separately for use with machines that require ROX. Maxima Hot Start *Taq* DNA polymerase in combination with an optimized buffer ensures PCR specificity and sensitivity. The SYBR Green I intercalating dye allows for DNA detection and analysis without using sequence-specific probes. dUTP is included in the mix for optional carryover contamination control using uracil-DNA glycosylase (UDG). The use of Maxima SYBR Green qPCR Master Mix in real-time PCR ensures reproducible, sensitive and specific quantification of genomic, plasmid, viral and cDNA templates.

Maxima Hot Start *Taq* DNA Polymerase is a *Taq* DNA polymerase which has been chemically modified by the addition of heat-labile blocking groups to amino acid residues. The enzyme is inactive at room temperature, avoiding extension of non-specifically annealed primers or primer dimers and providing higher specificity of DNA amplification. The enzyme provides the convenience of reaction set up at room temperature.

Maxima SYBR Green qPCR Buffer has been specifically optimized for qPCR analysis using SYBR® Green I. It contains both KCl and $(NH_4)_2SO_4$ to provide high specificity of primer annealing. The buffer composition allows for PCR at a wide range of $MgCl_2$ concentrations. Therefore, optimization of $MgCl_2$ concentration in PCR is generally not necessary.

SYBR Green I is a fluorescent intercalating dye which binds to the double stranded DNA and emits a fluorescent signal upon binding. In qPCR, DNA accumulates and fluorescent signal increases proportionally to the DNA concentration. The excitation and emission maxima of SYBR Green I are at 494 nm and 521 nm, respectively, which are compatible with the use on any real-time cycler.

dUTP is included in the master mix to partially replace dTTP in the accumulated PCR product, allowing for the option to prevent carryover contamination between reactions (1). Uracil-DNA Glycosylase (UDG) pre-treatment of the reaction removes all dU-containing amplicons carried over from previous reactions.

Note. UDG is not included in the Maxima SYBR Green qPCR Master Mix, no ROX, and must be purchased separately.

ROX Solution. ROX passive reference dye is supplied in a separate tube at 50 µM concentration. It can be added to a whole 2X master mix tube or to individual reaction mixture. ROX serves as an internal reference for normalization of the SYBR Green I fluorescent signal when using machines which can detect ROX, e.g. from Applied Biosystems. ROX allows for correction of well-to-well variation due to pipetting inaccuracies and fluorescence fluctuations. ROX does not participate in PCR and has a different emission spectrum (the excitation/emission maxima are at 580 nm/621 nm, respectively) compared to SYBR Green I. (Refer to a Table 1 to determine the recommended amounts of ROX concentrations for a specific instrument you use).

Table 1. Recommended amounts of ROX for a specific instrument.

Instrument	Amount of ROX per 25 µl reaction	Amount of ROX per 1.25 ml of 2X master mix	Final ROX concentration
Applied Biosystems: 7300, 7900HT, StepOne™, StepOnePlus™, ABI PRISM®7000 and 7700	0.05 µl	5 µl	100 nM
Applied Biosystems: 7500 **Stratagene:** Mx3000P™, Mx3005P™, Mx4000®	0.05 µl 10X diluted*	5 µl 10X diluted*	10 nM
Bio-Rad: iCycler® iQ, iQ5 and MyiQ™, Opticon®, CFX 96, CFX 384 **Roche:** LightCycler® 480, LightCycler® 2.0 **Corbett:** Rotor-Gene™ 3000, 6000 **Eppendorf:** MasterCycler™ ep *realplex* **Cepheid:** Smart Cycler®	Not required	Not required	Not required

* add 2 µl of ROX Solution to 18 µl of Water, nuclease-free, mix and use 0.05 µl for 25 µl qPCR reaction.

GUIDELINES TO ASSAY DESIGN

Templates

DNA. Genomic DNA up to 500 ng and plasmid DNA up to 10 ng can be used in qPCR with Maxima SYBR Green qPCR Master Mix, no ROX.

RNA. Template RNA for RT-qPCR must be free of DNA contamination. We recommend usage of DNase I, RNase-free (#EN0521), to remove trace amounts of DNA from RNA preparations. Always perform an RT-minus control to confirm complete removal of DNA (*see* below). For two-step RT-qPCR, up to 5 µg of total RNA can be used for cDNA synthesis in the reverse transcription reaction. An aliquot of the first strand cDNA synthesis reaction is then transferred to another tube as a template for qPCR.

The volume of the cDNA added (from the RT reaction) should not exceed 10% of the final qPCR volume.

For first strand cDNA synthesis, we recommend using our Maxima First Strand cDNA synthesis Kit for RT-qPCR, #K1641.

Primers

Primer design for qPCR is one of the most important factors to obtain efficient amplification and to avoid the formation of primer dimers.

Use primer design software, such as PrimerExpress® or Primer3 (frodo.wi.mit.edu) or follow general recommendations for PCR primer design below:

- GC content: 30-60%.
- Length: 18-30 nucleotides.
- Optimal amplicon length: 70-150 bp.
- Optimal melting temperature (Tm): 60°C. Differences in Tm of the two primers should not exceed 2°C.

- Avoid more than two G or C nucleotides in last five nucleotides at 3'-end to lower the risk of nonspecific priming.
- Avoid secondary structures in the amplicon.
- Avoid primer self-complementarities, complementarities between the primers and direct repeats in a primer to prevent hairpin formation and primer dimerization.
- Optimal primer concentration is 0.3 µM for both primers in most cases. The concentration may be optimized between 0.05 and 0.9 µM for individual primers and chosen by the lowest Ct for the amplicon and the highest Ct for primer-dimer formation (if present).

Necessary controls

- **No template control** (NTC) is important to assess for reagent contamination or primer-dimers. The NTC reaction contains all components except template DNA.
- **Reverse Transcriptase Minus (RT-) control** is important in all RT-qPCR experiments to assess for RNA sample contamination with genomic DNA. The control RT- reaction contains all components for RT-qPCR except the RT enzyme.

IMPORTANT NOTES

- Reaction set-up is at room temperature as the master mix includes Maxima Hot Start *Taq* DNA polymerase.
- We recommend a reaction volume of 25 µl. Other reaction volumes may be used if recommended for a specific instrument.
- Preparation of a master mix, which includes all reaction components except template DNA, helps to avoid pipetting errors and is an essential step in real-time PCR.
- Start PCR cycling with an initial denaturation step of 10 min at 95°C to activate Maxima Hot Start *Taq* DNA polymerase.
- Minimize exposure of Maxima SYBR Green qPCR Master Mix (2X), no ROX and ROX Solution to light during handling to avoid loss of fluorescent signal intensity.
- Readjust the threshold value for analysis of every run.
- When using Bio-Rad iCycler iQ or MyiQ systems collect well factors at the beginning of each experiment using an external well factor plate according to the instrument manufacturer's recommendations. Do not add fluorescein solution to the reaction mix. Well factors are used to compensate for any system or pipetting variations.

PROTOCOL

Reaction set-up

1. Gently vortex and briefly centrifuge all solutions after thawing.
2. Prepare a reaction master mix by adding the following components (except template DNA) for each 25 µl reaction to a tube at room temperature:

Maxima SYBR Green qPCR Master Mix (2X), no ROX*	12.5 µl
Forward Primer	0.3 µM**
Reverse Primer	0.3 µM**
ROX Solution	10 nM/ 100 nM***or not required
Template DNA	≤500 ng
Water, nuclease-free	to 25 µl
Total volume	25 µl

* Provides a final concentration of 2.5 mM $MgCl_2$.

** A final primer concentration of 0.3 µM is optimal in most cases, but may be individually optimized in a range of 0.05 µM to 0.9 µM.

*** see table 1 on p.3 for final concentration of ROX optimal for your instrument.

3. Mix the master mix thoroughly and dispense appropriate volumes into PCR tubes or plates.
4. Add template DNA (≤500 ng/reaction) to the individual PCR tubes or wells containing the master mix.

Note. For two-step RT-qPCR, the volume of the cDNA added from the RT reaction should not exceed 10% of the final PCR volume.

5. Gently mix the reactions without creating bubbles (do not vortex). Centrifuge briefly if needed. Bubbles will interfere with fluorescence detection.
6. Program the thermal cycler according to the recommendations below, place the samples in the cycler and start the program.

Thermal cycling conditions

Thermal cycling can be performed using a three-step or two-step cycling protocol.

Three-step cycling protocol

Step	Temperature, °C	Time	Number of cycles
Optional: UDG pre-treatment	50	2 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	30 s	
Extension	72	30 s	

Data acquisition should be performed during the extension step.

Two-step cycling protocol

Step	Temperature, °C	Time	Number of cycles
Optional: UDG pre-treatment	50	2 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing/Extension	60	60 s	

Data acquisition should be performed during the annealing/extension step.

Optional steps

- UDG pre-treatment. If using carryover decontamination, include a 2 min UDG digestion step at 50°C before the initial denaturation step.
- Melting curve analysis may be performed to verify the specificity and identity of the PCR product. Primer-dimers may occur during PCR if the primer design is not optimal. The dimers are distinguished from the specific product by a lower melting point.
- Agarose gel electrophoresis of PCR products. When designing a new assay it is recommended to verify the PCR product specificity by gel electrophoresis, as melting temperatures of a specific product and primer-dimers may overlap depending on the sequence composition.

TROUBLESHOOTING

Problem	Possible cause and solution
No amplification curve and no PCR product visible on a gel	PCR inhibitors present in the reaction mixture. Re-purify your template DNA. Primer design is suboptimal. Verify your primer design, use reputable primer design programs or validated pre-designed primers. RT-qPCR: inhibition by excess volume of the RT reaction. Volume of RT reaction product added to qPCR reaction should not exceed 10% of the total qPCR reaction volume. Pipetting error or missing reagent. Repeat the PCR reaction; check the concentrations of template and primers; ensure proper storage conditions of all reagents. Make new serial dilutions of template DNA or RNA. Degradation of primers. Check PCR primers for possible degradation on polyacrylamide gel. Annealing temperature is not optimal. Optimize the annealing temperature in 3°C increments. UDG present in PCR protocol with low annealing temperature. When performing UDG pre-treatment with conventional UDG, the temperature during PCR cycling should always be higher than 55°C. If annealing temperatures must be lower than 55°C, use heat-labile UDG.
No amplification curve but PCR product visible on a gel	qPCR instrument settings are incorrect. Check if instrument settings are correct (dye selection, reference dye, filters). Inactive fluorescence detection. Fluorescent detection should be activated and set at extension or annealing/extension step of the thermal cycling protocol. Instrument problems. Refer to the instrument manual for troubleshooting.
Amplification signal in non-template control	DNA contamination of reagents. • Follow general guidelines to avoid carry over contamination or include UDG pre-treatment step at the beginning of PCR. • Discard reagents and repeat with new reagents. RT-qPCR: RNA contaminated with genomic DNA. Design primers on intron/exon boundaries, treat RNA sample with DNaseI, RNA free (#EN0521) prior to reverse transcription. Primer-dimers. Use melting curve analysis to identify primer-dimers by the lower melting temperature compared to amplicon. If presence of dimers is confirmed: • Redesign your primers according to recommendations (see.p.3) or use validated pre-designed primers. • Optimize annealing temperature by increasing in 3°C increments.
Problem	Possible cause and solution
PCR efficiency is >110%	Non-specific products. Use melting curve analysis and gel electrophoresis to identify non specific amplicons. Optimize your primer design to avoid such artifacts or use validated pre-designed primers.

Problem	Possible cause and solution
PCR efficiency is <90%	PCR inhibitors present in a reaction mixture. Re-purify your template DNA. PCR conditions are suboptimal. Verify the primer concentrations. Verify storage conditions of qPCR master mix. Primer design. Verify your primer design, use primer design programs or validated pre-designed primers. Avoid designing primers in regions with high DNA secondary structure.
Poor standard curve	Excessive amount of template. Do not exceed maximum recommended amounts of template DNA (500 ng DNA for 25 µl reaction). Suboptimal amount of template. Increase the amount of template, if possible. RT-qPCR: inhibition by excess volume of the RT reaction. Volume of RT reaction product added to qPCR reaction should not exceed 10% of the total qPCR reaction volume.
Non-uniform fluorescence intensity	Contamination of the thermal cycler. Perform decontamination of your real-time cycler according to the supplier's instructions. Poor calibration of the thermal cycler. Perform calibration of the real-time cycler according to the supplier's instructions.

REFERENCE

1. Longo, M.C., et al., Use of uracil DNA glycosylase to control carryover contamination in polymerase chain reactions, Gene, 93, 125-128, 1990.

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