Thermo SCIENTIFIC

PRODUCT INFORMATION

Thermo Scientific
Luminaris HiGreen Low ROX qPCR Master Mix

#K0974
For 5000 rxns of 20 µL
Lot _____
Expiry Date __
Store at -20 °C in the dark

www.thermoscientific.com/onebio

CERTIFICATE OF ANALYSIS

The absence of endo-, exodeoxyribonucleases and ribonucleases confirmed by appropriate quality tests.
Functionally tested in real-time PCR in parallel 20 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

Rev.1

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COMPONENTS

Component	**#K0971** for 250 rxns of 20 µL	**#K0972** for 500 rxns of 20 µL	**#K0973** for 1250 rxns of 20 µL	**#K0974** for 5000 rxns of 20 µL
Luminaris HiGreen Low ROX qPCR Master Mix (2X)	2 × 1.25 mL	4 × 1.25 mL	10 × 1.25 mL	4 × 12.5 mL
Water, nuclease-free	2 × 1.25 mL	4 × 1.25 mL	10 × 1.25 mL	2 × 30 mL

STORAGE

Store the master mix at -20°C for long term storage or at 4°C for up to one month. SYBR® Green I and ROX dyes in the master mix are sensitive to light and should be protected from direct light. When stored at -20°C, full activity of the mix is retained for at least 24 months as indicated on the tube label.

DESCRIPTION

Thermo Scientific Luminaris HiGreen Low ROX qPCR Master Mix (2X) is a ready-to-use solution optimized for quantitative real-time PCR (qPCR) and two-step RT-qPCR. The master mix includes Hot Start *Taq* DNA polymerase, uracil-DNA glycosylase (UDG) and dNTPs in an optimized PCR buffer. It contains double-stranded DNA (dsDNA) binding dye and is supplemented with ROX passive reference dye. Only the template and primers need to be added. Hot Start *Taq* DNA polymerase in combination with an optimized buffer ensures PCR specificity and sensitivity. The dsDNA binding dye SYBR Green I allows for DNA detection and analysis without using sequence-specific probes. dUTP and UDG are included in the mix for carryover contamination control.
The use of Luminaris HiGreen Low ROX qPCR Master Mix in qPCR ensures reproducible, s ensitive and specific quantification of genomic, plasmid, viral and cDNA templates.
The master mix can be used with real-time thermal cyclers that are compatible with low concentration of ROX reference dye; Applied BioSystems: ABI PRISM® 7500, ViiA™ 7, Stratagene: Mx3000P™, Mx3005P™, Mx4000®.

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 the extension of non-specifically annealed primers or primer dimers and providing higher specificity of DNA amplification. The enzyme provides the convenience of reaction setup at room temperature.

Uracil-DNA Glycosylase and **dUTP** are included in the master mix to prevent carryover contamination between reactions (1). UDG pre-treatment of the reaction removes all dU-containing amplicons carried over from previous reactions.

HiGreen 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. As DNA accumulates, the 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.

ROX Passive Reference Dye is included in the master mix to serve as an internal reference for normalization of the fluorescent signal when carrying out reactions in instrumets that can detect ROX, such as Applied BioSystems, Stratagene. ROX allows for correction of well-to-well variation appearing due to pipetting inaccuracies and fluorescence fluctuations. The presence of ROX does not interfere with qPCR using other systems, such as iCycler iQ, since it 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.

GUIDELINES TO ASSAY DESIGN

Templates. Template amount depends on the type and quality of the template.

DNA. Genomic DNA up to 200 ng and plasmid DNA up to 10 ng can be used in a 20 µL qPCR reaction with Luminaris HiGreen Low ROX qPCR Master Mix. Note that plasmid copy number in 1 µg of plasmid DNA is equivalent to 9.1×10^{11} divided by the plasmid size in kilobases.

cDNA. For the first strand cDNA synthesis, we recommend Thermo Scientific Maxima First Strand cDNA Synthesis Kit for RT-qPCR, #K1641.

The volume of the cDNA added (from the RT reaction) to the qPCR reaction with Luminaris HiGreen Low ROX qPCR Master Mix should not exceed 10% of the final reaction volume. If high-abundance genes are to be detected, we recommend preparing a dilution series of the cDNA template prior to qPCR for the most accurate results. Then add diluted cDNA up to 10% of qPCR volume.

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 the 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 the last five nucleotides at 3' end to lower the risk of nonspecific priming.
- Avoid secondary structures in the amplicon.
- Avoid self-complementarities in a primer, complementarities between the primers and direct repeats in a primer to prevent hairpin formation and primer dimerization.
- Optimal primer concentration in qPCR reaction is 0.3 µM for each primer in most cases. The concentration may be optimized between 0.05 and 0.9 µM for individual primers and chosen by the lowest quantification cycle (Cq) for the amplicon and the highest Cq 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 should contain all components except template DNA.
- **Reverse Transcriptase Minus (RT-) control** is important in all RT-qPCR experiments to assess for RNA sample contamination with DNA. This control reaction should be performed during the first strand cDNA synthesis by combining all components for reverse transcription except the RT enzyme. Afterwards, a sample of control RT- reaction is added to a qPCR reaction, up to 10% of qPCR reaction volume.

IMPORTANT NOTES

- The reaction setup can be performed at room temperature. The initial denaturation step in the PCR protocol reactivates the Hot Start *Taq* DNA polymerase.
- We recommend a reaction volume of 20 µL. Other reaction volumes may be used if recommended for a specific instrument. The minimum reaction volume depends on the real-time instrument and consumables (follow the supplier's recommendations). The reaction volume can be increased if a high template amount is used.
- Preparation of a master mix, which includes all reaction components except template DNA, helps to avoid pipetting errors and is an essential step in qPCR.
- Start PCR cycling with the UDG treatment step of 2 min at 50°C followed by an initial denaturation step of 10 min at 95°C to activate Hot Start *Taq* DNA polymerase.
- Minimize the exposure of Luminaris HiGreen Low ROX qPCR Master Mix (2X) to light during handling to avoid the loss of fluorescent signal intensity.
- Adjust the threshold value for analysis of every run.
- When using the Bio-Rad iCycler iQ or MyiQ systems, collect the 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 setup

1. Gently vortex and briefly centrifuge all solutions after thawing.
2. Calculate all components required for appropriate qPCR volume. See recommendations in Table 1.

Table 1. Reaction setup:

Components (in order of addition)	10 µL rxn	20 µL rxn	50 µL rxn	Final concentration
Master Mix (2X)*	5 µL	10 µL	25 µL	1X
10 µM Forward Primer	0.3 µL	0.6 µL	1.5 µL	0.3 µM**
10 µM Reverse Primer	0.3 µL	0.6 µL	1.5 µL	0.3 µM**
Template DNA	X µL	X µL	X µL	Do not exceed 10 ng/µL in the final reaction
Water, nuclease-free	add to 10 µL	add to 20 µL	add to 50 µL	

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

** 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.

3. Prepare the reaction master mix by adding the Master Mix (2X), Primers and Water for each qPCR reaction to a tube at room temperature.
4. Mix the master mix thoroughly and dispense appropriate volumes into PCR tubes or plates.
5. Add template DNA (≤ 200 ng/reaction for genomic DNA or ≤ 10 ng/reaction for plasmid DNA) to the individual PCR tubes or plates 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 qPCR volume.

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

- **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.

Note. If agarose gel electrophoresis or cloning of qPCR products is going to be performed, after cycling, store the qPCR reactions at -20ºC for long term or at +4ºC for up to 2 days. This is to avoid PCR product degradation by UDG, which gains back its activity when the qPCR mix cools below 55ºC.

TROUBLESHOOTING

Problem	Possible cause and solution
No amplification curve and no PCR product visible on a gel	**PCR inhibitors present in the reaction mixture.** Repurify 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 the excess volume of RT reaction.** The volume of RT reaction 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 cDNA synthesis reaction. **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 a PCR protocol with low annealing temperature.** Due to the presence of UDG in the Luminaris HiGreen Low ROX qPCR Master Mix, the temperature during PCR cycling should always be higher than 55°C.
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 no template control	**DNA contamination of reagents.** • Follow general guidelines to avoid carryover contamination. • Discard used reagents and repeat with new reagents. **RT-qPCR: RNA contaminated with genomic DNA.** Design primers on intron/exon boundaries, treat RNA sample with DNaseI, RNase-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.
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.** Repurify 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 (200 ng DNA for 20 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. M. C. Longo, *et al*., Use of uracil DNA glycosylase to control carryover contamination in polymerase chain reactions, *Gene* **93**, 125-128 (1990).

NOTICE TO PURCHASER:

- Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 6,127,155, 5,677,152 (claims 1 to 23 only) and 5,773,258 (claims 1 and 6 only). Use of this product in a passive reference method is covered by the following U.S. Patent: 5,928,907 (claim numbers 12-24, 27-28) and corresponding patent claims outside the US. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.
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Please refer to www.thermoscientific.com/onebio for Material Safety Data Sheet of the product.