



PRODUCT INFORMATION

Thermo Scientific

Luminaris Color HiGreen Low ROX qPCR Master Mix

#K0371

For 250 rxns of 20 μ L

Lot _____

Exp. __

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 μ L 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

CONTENTS

| | page |
|----------------------------------|------|
| COMPONENTS | 2 |
| STORAGE..... | 2 |
| DESCRIPTION..... | 2 |
| GUIDELINES TO ASSAY DESIGN | 3 |
| Templates | 3 |
| Primers | 4 |
| Necessary controls..... | 5 |
| IMPORTANT NOTES..... | 5 |
| PROTOCOL..... | 6 |
| Reaction setup | 6 |
| Thermal cycling conditions..... | 6 |
| Optional steps | 7 |
| TROUBLESHOOTING..... | 8 |
| REFERENCE..... | 9 |
| NOTICES..... | 10 |

COMPONENTS

| Component | #K0371 for 250 rxns of 20 µL | #K0372 for 500 rxns of 20 µL | #K0373 for 1250 rxns of 20 µL | #K0374 for 5000 rxns of 20 µL |
|--|------------------------------------|------------------------------------|-------------------------------------|-------------------------------------|
| Luminaris Color HiGreen Low ROX qPCR Master Mix (2X) | 2 × 1.25 mL | 4 × 1.25 mL | 10 × 1.25 mL | 4 × 12.5 mL |
| 40X Yellow Sample Buffer | 1 × 1.25 mL | 1 × 1.25 mL | 3 × 1.25 mL | 1 × 13 mL |
| Water, nuclease-free | 2 × 1.25 mL | 4 × 1.25 mL | 10 × 1.25 mL | 2 × 30 mL |

STORAGE

Store at -20 °C in the dark for long term storage or at 4 °C for up to one month.

DESCRIPTION

Thermo Scientific Luminaris Color HiGreen Low ROX qPCR Master Mix (2X) is a universal ready-to-use solution optimized for quantitative real-time PCR (qPCR) and two-step RT-qPCR on most real-time PCR instruments.

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 Luminaris™ Color HiGreen Low ROX qPCR Master Mix is supplemented with an inert blue dye and a separate Yellow Sample Buffer that contains a yellow dye. Mixing both components in a qPCR reaction turns the solution green. This provides a visual aid when pipetting and decreases the risk of pipetting errors during reaction setup, especially when using white reaction vessels. The dyes do not affect the specificity or sensitivity of qPCR assays.

The use of Luminaris Color HiGreen Low ROX qPCR Master Mix in qPCR ensures reproducible, sensitive 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 (UDG) 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 (NH₄)₂SO₄ to provide high specificity of primer annealing. The buffer composition allows for PCR at a wide range of MgCl₂ concentrations. Therefore, optimization of MgCl₂ 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 cyclers.

An inert blue dye helps keeping track of pipetting of the master mix into the reaction wells. It is easy to monitor which wells in a PCR plate are empty and which ones already contain the blue master mix. The absorption maximum of the blue dye is at 615 nm.

40X Yellow Sample Buffer is provided with Luminaris Color HiGreen Low ROX qPCR Master Mix. It is used to color and track the samples. When using the blue master mix the PCR reaction mix is blue before sample addition. After adding the sample the reaction mix turns green, making it easy to track the pipetting of samples. The buffer is provided as a 40X concentrate and used in 1X concentration in the final reaction. Using the Yellow Sample Buffer is optional. The absorption maximum of the yellow dye is at 413 nm.

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 instruments 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 Color HiGreen Low ROX qPCR Master Mix. Note that plasmid copy number in 1 µg of plasmid DNA equals to 9.1×10^{11} divided by the plasmid size in kilobases.

If using the Yellow Sample Buffer (optional), add buffer to the samples to a concentration that will yield 1X in the final reaction volume. For example, if 5 µL of the sample is to be used in a 20 µL reaction volume, add Yellow Sample Buffer to obtain 4X buffer concentration in the sample for 1X buffer concentration in the final reaction. A 4Xconcentrated Yellow Sample Buffer stock could be prepared by diluting 10 µL of 40X Yellow Sample Buffer with nuclease-free water to 100 µL, see recommendations in Table 1.

Table 1. Yellow Sample Buffer concentration in the sample, when different amount of sample is to be used in a final qPCR reaction of 20 µL:

| Sample volume to be added to a qPCR reaction (20 µL) | 1 µL | 2 µL | 2.5 µL | 3 µL | 4 µL | 5 µL | 6 µL | 7 µL | 8 µL |
|---|-------|-------|--------|---------|---------|-------|--------|--------|--------|
| Yellow Sample Buffer concentration needed in the sample | 20X | 10X | 8X | 6.7X | 5X | 4X | 3.3X | 2.9X | 2.5X |
| Volume of 40X Yellow Sample Buffer in 100 µL of sample, giving the final concentration needed in the sample | 50 µL | 25 µL | 20 µL | 16.7 µL | 12.5 µL | 10 µL | 8.4 µL | 7.2 µL | 6.3 µL |

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 Color 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.

If using the Yellow Sample Buffer (optional), add 5 µL of 40X Yellow Sample Buffer to 20 µL cDNA synthesis reaction, then use 2.5 µL of the mix (this will comprise 2 µL of cDNA and 0.5 µL of Yellow Sample Buffer) into a 20 µL qPCR reaction.

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 (T_m): 60 °C. Differences in T_m 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 (C_q) for the amplicon and the highest C_q 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 μ L. 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 Color HiGreen Low ROX qPCR Master Mix (2X) to light during handling to avoid the loss of fluorescent signal intensity.
- Readjust 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 2.

Table 2. Reaction setup:

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

* Provides a final concentration of 2.5 mM MgCl₂.

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

3. Prepare the reaction master mix by adding the 2X Master Mix, 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 (\leq 200 ng/reaction) 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 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 |
|----------------------|-----------------|--------|------------------|
| 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 -20 °C for long term, or at +4 °C 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 55 °C.

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. |
| No amplification curve but PCR product visible on a gel | 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 Color HiGreen Low ROX qPCR Master Mix, the temperature during PCR cycling should always be higher than 55 °C. |
| | qPCR instrument settings are incorrect. Check if instrument settings are correct (dye selection, reference dye, filters). |
| Amplification signal in no template control | 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. |
| | DNA contamination of reagents. <ul style="list-style-type: none"> • 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. |
| PCR efficiency is > 110% | Primer dimers. Use melting curve analysis to identify primer dimers by the lower melting temperature compared to amplicon. If presence of dimers is confirmed: <ul style="list-style-type: none"> • Redesign your primers according to recommendations (<i>see p.4</i>) or use validated pre-designed primers. Optimize annealing temperature by increasing in 3 °C increments. |
| | 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% | <p>PCR inhibitors present in a reaction mixture. Repurify your template DNA.</p> <p>PCR conditions are suboptimal. Verify the primer/probe concentrations. Verify storage conditions of qPCR master mix.</p> <p>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.</p> |
| Poor standard curve | <p>Excessive amount of template. Do not exceed maximum recommended amounts of template DNA (200 ng DNA for 20 µL reaction).</p> <p>Suboptimal amount of template. Increase the amount of template, if possible.</p> <p>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.</p> |
| Non-uniform fluorescence intensity | <p>Contamination of the thermal cycler. Perform decontamination of your real-time cycler according to the supplier's instructions.</p> <p>Poor calibration of the thermal cycler. Perform calibration of the real-time cycler according to the supplier's instructions.</p> |

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.

NOTICE TO PURCHASER: LIMITED LICENSE

- 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), 5,994,056, 6,171,785. 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.
- This product is provided under an agreement between Molecular Probes, Inc. and Thermo Fisher Scientific, Inc. and the manufacture, use, sale or import of this product is subject to one or more of U.S. Patents, and corresponding international equivalents, owned by Molecular Probes, Inc. (a wholly-owned subsidiary of Invitrogen Corp. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer, where such research does not include testing, analysis or screening services for any third party in return for compensation on a per test basis. The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. For information on purchasing a license to this product for purposes other than research, contact Molecular Probes, Inc., Business Development, 29851 Willow Creek Road, Eugene, OR 97402, USA Tel: (541) 465-8300, Fax: (541) 335-0354.
- The purchase of this product includes a limited, nontransferable license, under specific claims of one or more U.S. patents owned by the University of Utah Research Foundation and/or Idaho Technology, Inc., to use only the enclosed amount of product according to the specified protocols. No right is conveyed, expressly, by implication, or by estoppel, to use any instrument or system under any claim of such U.S. patent(s), other than for the amount of product contained herein.
- This product or the use of this product is covered by US patent application US20110251080A1 and corresponding counterparts. The purchase of this product includes a non-transferable license to use this product for the purchaser's internal research. All other commercial uses of this product, including without limitation product use for diagnostic purposes, resale of product in the original or any modified form or product use in providing commercial services require a separate license. For further information on obtaining licenses please contact ltvil.info@thermofisher.com

PRODUCT USE LIMITATION

This product is developed, designed and sold exclusively for research purposes and *in vitro* use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.

Please refer to www.thermoscientific.com/onebio for Material Safety Data Sheet of the product.

© 2012 Thermo Fisher Scientific, Inc. All rights reserved. SYBR is a registered trademark of Molecular Probes, Inc. ABI PRISM, ROX, ViiA and PrimerExpress are registered trademarks of Applied Biosystems, Inc. Mx3000P, Mx3005P are registered trademarks and Mx4000 is trademark of Stratagene, Inc. All other trademarks are the property of Thermo Fisher Scientific Inc. and its subsidiaries.