PRODUCT INFORMATION

Thermo Scientific Luminaris Color Probe qPCR Master Mix

#K0353

For 1250 rxns of 20 µL
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 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	**#K0351** for 250 rxns of 20 µL	**#K0352** for 500 rxns of 20 µL	**#K0353** for 1250 rxns of 20 µL	**#K0354** for 5000 rxns of 20 µL
Luminaris Color Probe 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 Probe 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. Hot Start *Taq* DNA polymerase in combination with an optimized buffer ensures PCR specificity and sensitivity. dUTP and UDG are included in the mix for carryover contamination control. The Luminaris™ Color Probe 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 Probe qPCR Master Mix in qPCR ensures reproducible, sensitive and specific quantification of genomic, plasmid, viral and cDNA templates.

ROX Solution can be purchased separately (#R1371) for use with instruments that require ROX. Also, Luminaris Color Probe High ROX qPCR Master Mix (#K0331/2/3/4) and Luminaris Color Probe Low ROX qPCR Master Mix (#K0341/2/3/4) are formulated specifically for use with the instruments requiring high ROX and low ROX, respectively.

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.

Probe qPCR Buffer has been specifically optimized for qPCR analysis using sequence-specific probes. 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.

An inert blue dye helps keep 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 Probe 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 concentration 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.

The excitation and emission spectra of most commonly used fluorophores and quenchers reside in a visible range of 495–670 nm. The blue dye in the master mix and the yellow dye in the sample buffer provided with the Luminaris Color Probe qPCR Master Mix absorb at 615 nm and 413 nm, respectively. Typically, the excitation or emission spectrum of common qPCR fluorophores are not at these wavelengths. However, dyes that are excited or emit close to these wavelengths, such as ROX and Texas Red, can appear less intensive than in the reactions without the colored components, but this does not affect the specificity or sensitivity of qPCR assays.

ROX Solution, 50 µM can be purchased separately (#R1371) for use with instruments that require ROX. It can be added to an entire 2X master mix tube or to an individual reaction mixture. ROX serves as an internal reference for normalization of fluorescent signal when using instruments which can detect ROX, such as from Applied BioSystems. ROX allows for the 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 dyes used for probes. (Refer to Table 1 to determine the recommended amounts of ROX Solution for a specific instrument).

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

Instrument	Amount of ROX per 20 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	0.14 µL	17.5 µL	350 nM
Applied Biosystems: 7500, ViiA™ 7 **Stratagene:** Mx3000P™, Mx3005P™, Mx4000®	0.14 µL 10X diluted*	17.5 µL 10X diluted*	35 nM
Thermo Scientific: PikoReal Real-Time PCR System **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 50 µM ROX Solution to 18 µL of Water, nuclease-free, mix and use 0.14 µL for 20 µL qPCR reaction.

Luminaris Color Probe High ROX qPCR Master Mix (#K0331/2/3/4) and Luminaris Color Probe Low ROX qPCR Master Mix (#K0341/2/3/4) are qPCR master mixes formulated specifically for use with the instruments requiring high ROX and low ROX, respectively.

GUIDELINES TO ASSAY DESIGN

Templates.

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

DNA. Genomic DNA (≤ 200 ng) and plasmid DNA (≤ 10 ng) can be used in a 20 µL qPCR reaction with Luminaris Color Probe 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 4X concentrated 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 2.

Table 2. 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 Probe 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 (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).

Probes

For probe design follow the general recommendations for primer design described above.
Design the probe first, and then select the primers that flank the probe region.
General guidelines for design of dual-labeled probes, such as hydrolysis probes:

- GC content: 30-60%.
- Length: 20-30 bases.
- Melting temperature (Tm): 68-70 °C, 8-10 °C higher than the Tm of the PCR primers.
- Avoid runs of the same nucleotide, especially guanine, longer than 3 bases.
- Exclude G at the 5' end of the probe, which causes quenching.
- Select the template DNA strand containing more C than G bases.
- Avoid secondary structures.
- Avoid dimerization with primers.

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 reverse transcription 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 minutes at 50 °C followed by an initial denaturation step of 10 minutes at 95 °C to activate Hot Start *Taq* DNA polymerase.
- 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 set-up

1. Gently vortex and briefly centrifuge all solutions after thawing.
2. Calculate all components required for appropriate qPCR volume. See recommendations in Table 3.

Table 3. Reaction setup:

Components (in order of addition)	**10 µLrxn**	**20 µL rxn**	**50 µL rxn**	**Final concentration**
2X Master Mix*	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**
10 µM Probe	0.2 µL	0.4 µL	1 µL	0.2 µM
Template DNA (including Yellow Sample Buffer, optional)	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 a final concentration of 4 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.

3. Prepare the reaction master mix by adding the 2x Master Mix, Primers, Probe 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) to the 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 annealing 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

- **Agarose gel electrophoresis of PCR products.** When designing a new assay it is recommended to verify the PCR product specificity by gel electrophoresis.

Note. If agarose gel electrophoresis or cloning of qPCR products is going to be performed, after cycling store the qPCR reactions at -20 ºC for long term, or at +4 ºC 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 ºC.

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 Color Probe 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 annealing or annealing/extension step of the thermal cycling protocol. **Instrument problems.** Refer to the instrument manual for troubleshooting. **Degradation of probe.** Check the probe for possible degradation on polyacrylamide gel.
Amplification signal in no template control	**DNA contamination of reagents.** • Follow general guidelines to avoid carry-over contamination. • Discard used reagents and repeat with new reagents. **RT-qPCR: RNA contaminated with genomic DNA.** Design primers or probe on intron/exon boundaries, treat RNA sample with DNaseI, RNase-free (#EN0521) prior to reverse transcription.
PCR efficiency is > 110%	**Non-specific products.** Use 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/probe 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. Longo, M.C., et al., Use of uracil DNA glycosylase to control carryover contamination in polymerase chain reactions, Gene, 93, 125-128, 1990.

NOTICES

- 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), 5,773,258 (claims 1 and 6 only). 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 seperate license from Roche. Further information on purchasing licenses may be obtained from the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.
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PRODUCT USE LIMITATION

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