Thermo SCIENTIFIC

PRODUCT INFORMATION

Thermo Scientific
Luminaris Color HRM Master Mix

#K1031

For 250 reactions of 20 µL

Lot _____

Exp. __

Store at -20 °C in the dark

www.thermoscientific.com/onebio

CERTIFICATE OF ANALYSIS

Functionally tested to demonstrate detection of various SNPs by HRM analysis. The absence of endo-, exodeoxyribonucleases and ribonucleases confirmed by appropriate quality tests.

Quality authorized by:  Jurgita Zilinskiene

Rev.1. 69

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COMPONENTS

Component	#K1031 for 250 rxns of 20 µL	#K1032 for 1250 rxns of 20 µL
Luminaris Color HRM Master Mix (2X)	2 × 1.25 mL	10 × 1.25 mL
40X Yellow Sample Buffer	1 × 1.25 mL	3 × 1.25 mL
Water, nuclease free	2 × 1.25 mL	10 × 1.25 mL

STORAGE

Store at -20 °C in the dark for long term storage or at 4 °C for up to three weeks. When stored at -20 °C, full activity of the mix is retained for at least 24 months as indicated on the tube label. Repeated freezing and thawing of the Master Mix is not recommended.

DESCRIPTION

Thermo Scientific™ Luminaris™ Color HRM Master Mix is a convenient, ready-to-use master mix designed for the accurate detection of DNA sequence variations using High Resolution Melt (HRM) analysis.

The Luminaris Color HRM Master Mix (2X) contains Hot Start Taq DNA polymerase in an optimized buffer system with EvaGreen™ fluorescent dye for robust and reliable performance in PCR and HRM. The chemically modified Hot Start Taq DNA polymerase has ultra low residual activity until thermally activated ensuring PCR specificity and sensitivity.The DNA binding dye EvaGreen enables discrimination of even the most challenging sequence differences (such as Type IV SNP) without PCR inhibition and with no apparent sequence preference.

The Luminaris Color HRM 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 errors during reaction setup, especially when using white reaction vessels. The dyes do not affect the specificity, sensitivity or PCR product discrimination in HRM assays.

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.

EvaGreen™ dye is a third-generation fluorescent dye that selectively binds to double-stranded DNA (dsDNA). In qPCR, EvaGreen can be used at a much higher inhibition-free concentration, resulting in more robust PCR signal which suits ideally for HRM. The excitation and emission maxima of EvaGreen dye are at 500 nm and 530 nm, respectively, in DNA-bound state, and 471 nm without DNA, which is compatible with the use on any real-time instrument.

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 HRM Master Mix. It is used to stain 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.

Luminaris Color HRM Master Mix has been optimized for use with the Thermo Scientific™ PikoReal™ 96 Real-Time PCR System, Bio-Rad CFX96™, QIAGEN Rotor-Gene™ 6000, Applied Biosystems 7500 Fast System and Viia™ 7, and Roche LightCycler®480 real-time instruments.

GUIDELINES TO ASSAY DESIGN

Template

Use any template DNA (e.g., genomic DNA) suitable for PCR.

For optimal results, use 100 pg-20 ng of genomic DNA per 10-20 µL reaction. Applying DNA concentrations outside the recommended range may increase the non-specific amplification and produce erroneous HRM results.

For best results, all DNA templates should be prepared using the same method and eluted/diluted in the same buffer, as the variation in salt concentration strongly affects melting properties of DNA. Additionally, the volume and concentration of template DNA added to PCR reactions should be kept as similar as possible.

The amplification of samples analyzed using the same primer system should not differ by more than two Cq values. Note, that assays resulting in Cq values above 30 should be analyzed with care.

Sample staining with the Yellow Sample Buffer provides an option for visual tracking of pipetting during the reaction setup.

Add Yellow Sample 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 a 1X buffer concentration in the final reaction. A 4X concentrated Yellow Sample Buffer stock can be prepared by diluting 5 µL of 40X Yellow Sample Buffer with nuclease-free water up to 50 µL. See recommendations in Table 1 when preparing stained DNA samples for reaction volume of 20 µL, and Table 2, when final reaction volume is 10 µL.

Important note. In order to get accurate HRM results the concentration of DNA and Yellow Sample Buffer in all DNA standards and samples of the same run should be as similar as possible.

The yellow-stained DNA standards can be stored at -20 °C for up to 1 month.

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

Sample volume to be added to a HRM 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 50 µL of sample, giving the final concentration needed in the sample	25 µL	13 µL	10 µL	8.4 µL	6.3 µL	5 µL	4.2 µL	3.6 µL	3.2 µL

Table 2. Yellow Sample Buffer concentration in the sample when different amount of sample is to be used in a final HRM reaction of 10 µL:

Sample volume to be added to a HRM reaction (10 µL)	1 µL	2 µL	2.5 µL	3 µL	4 µL
Yellow Sample Buffer concentration needed in the sample	10X	5X	4X	3.3X	2.5X
Volume of 40x Yellow Sample Buffer in 50 µL of sample, giving the final concentration needed in the sample	13 µL	6.3 µL	5 µL	4.2 µL	3.2 µL

Primers and assay design

For optimal SNP detection, design primers to produce 50–200 bp long PCR amplicons. Typically, primers producing shorter amplicons can better distinguish genotypes for a SNP, especially for Type III and Type IV SNPs. In longer fragments the risk of covering multiple mutations is also increased.

We recommend using primer design software, such as Primer Express® 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 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.

Start with the final primer concentration of 0.5 µM for each primer.

Using HPLC-purified primers is not essential, but may help in analysis of complicated or longer amplicons with Type IV SNP when salt concentration becomes critical.

Avoid non-specific amplification products generated during PCR, as it greatly affects the quality of HRM results.

Necessary controls

No-template control (NTC) is important to assess for reagent contamination or primer-dimers. Prepare at least one NTC reaction containing all components except for template DNA.

Positive control reactions are necessary for each expected genetic variant. Use positive controls with known sequence to avoid multiple mutations and undesirable HRM profiles. At least one positive control reaction per genotype is recommended.

HRM analysis

It is recommended to perform HRM analysis as soon as possible following the PCR amplification. For the data analysis, follow recommendations provided by the supplier of HRM software.

When performing HRM analysis for the first time it is recommended in the melt curve/dissociation step to span a wide range of temperatures (eg. 55-95 °C) to ensure covering all possible melting points. If applicable, use increments of 0.02 °C/s.

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 reaction volumes between 10 µL and 20 µL. For PikoReal 96 Real-Time PCR System a reaction volume of 10 µL should be used. For other real-time instruments the minimum reaction volume depends on the specific instrument and consumables (follow the supplier's recommendations).
- We recommend performing reactions in white plastic plates for increased sensitivity and accuracy of the HRM results.
- Preparation of a master mix, which includes all reaction components except template DNA, helps to avoid pipetting errors and is an essential step in PCR and HRM.
- Minimize the exposure of Luminaris Color HRM Master Mix (2X) to light during handling to avoid the loss of fluorescent signal intensity.

PROTOCOL

Reaction setup

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

Table 3. Reaction setup:

Components (in order of addition)	10 µL reaction	20 µL reaction	Final concentration
Master Mix (2X)*	5 µL	10 µL	1X
10 µM Forward Primer	0.5 µL	1.0 µL	0.5 µM**
10 µM Reverse Primer	0.5 µL	1.0 µL	0.5 µM**
Template DNA (including Yellow Sample Buffer, optional)	X µL	X µL	Do not exceed 20 ng in the final volume
Water, nuclease-free	add to 10 µL	add to 20 µL	

* Provides $MgCl_2$ at final concentration of 3.5 mM.

** A final primer concentration of 0.5 µM is optimal in most cases, but may be individually optimized in a range of 0.3 µM to 0.9 µM.

3. Prepare the reaction master mix by adding the Master Mix (2X), primers and water for each PCR reaction to a tube at room temperature.
4. Mix reaction master mix thoroughly and dispense appropriate volumes into PCR tubes or plates.
5. Add template DNA (≤ 20 ng/reaction) to the individual PCR tubes or plates containing the master mix.
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 two-step or three-step cycling protocol.
It is recommended to program the PCR cycling using the maximal ramp rate available.

Two-step cycling protocol

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	95	10 min	1
Denaturation	95	10 sec	35-45
Annealing/Extension*	60	60 sec	
Heteroduplex formation (optional) **	95	30 sec	1
	50	30 sec	1
Melt curve/dissociation***	65-95	Increments of 0.2 °C/2s Data acquisition	

* Data acquisition should be performed during the annealing/extension step.
** Performing Heteroduplex formation step is optional; in some cases this may improve HRM results.
*** The temperature range of the melt curve/dissociation step and size of temperature increments can be optimized depending on the melting of the amplicon.

Three-step cycling protocol

Three-step cycling protocol is recommended for complicated (especially Type IV SNP) or longer than 200 bp amplicons.

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	95	10 min	1
Denaturation	95	10 sec	35-45
Annealing	60	30 sec	
Extension*	72	30 sec	
Heteroduplex formation (optional) **	95	30 sec	1
	50	30 sec	1
Melt curve/dissociation***	65-95	Increments of 0.2 °C/2s Data acquisition	

* Data acquisition should be performed during the extension step.
** Performing Heteroduplex formation step is optional; in some cases this may improve HRM results.
*** The temperature range of the melt curve/dissociation step and size of temperature increments can be optimized depending on the melting of the amplicon.

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.

TROUBLESHOOTING

Problem	Possible cause and solution
Late amplification: Cq value >30	**The amount of DNA added to HRM reaction is too low.** Increase sample input or the number of amplification cycles. **DNA purity problem.** Re-purify your template DNA. **Annealing temperature is not optimal.** Optimize the annealing temperature in 1 °C increments. **Tested amplicon is longer than 250 bp**. Increase the extension time.
No amplification	**PCR inhibition.** Re-purify your template DNA. **Pipetting error or missing reagent.** Repeat the PCR reaction; check the concentration of template and primers; ensure proper storage conditions of all reagents. **Annealing temperature is not optimal.** Optimize the annealing temperature in 1 °C increments. **Primer design is suboptimal.** Re-design primers.
Non-specific amplification	**Primer design is suboptimal.** Verify your primer design, use reputable primer design programs or validated pre-designed primers. **Multiple non-specific targets are amplified.** Increase the annealing temperature. After PCR amplification, run PCR product on a gel to make sure that it contains a single band. Reduce the number of amplification cycles.
HRM curves of sample replicates are widely spread	**Low PCR efficiencies.** Ensure efficient PCR. **Concentrations of template are very different.** Make sure that the starting concentrations of tested DNA samples are similar. **Variations in salt concentration.** Check the purity of the template solution. Harmonize the template solutions by diluting the samples in the same buffer.
Multiple melting peaks	**Too long amplicon.** Re-design the primers to make amplicon shorter. **More than one SNP in the amplicon (genotyping experiments only).** Re-design the primers so that the amplicon contains only 1 SNP.

Problem	Possible cause and solution
Amplification signal in no-template control	**DNA contamination of reagents.** Follow general guidelines of good laboratory practice. Discard used reagents and repeat reactions with new reagents. **Primer-dimers.** Use melting curve analysis to identify primer-dimers by the lower melting temperature compared to amplicon. If the presence of dimers is confirmed, re-design your primers according to recommendations (*see* p.6) or use validated pre-designed primers. Optimize annealing temperature by increasing in 1 °C increments.
Different genotypes, particularly between homozygotes, cannot be detected during HRM analysis	**Difficult to detect mutation (usually Type III or IV SNP)** Amplicon is too long; re-design the primers to produce shorter amplicon. Increase sample input or the number of amplification cycles. Use DNA samples colored with the Yellow Sample Buffer. Use wider temperature range in melt curve/dissociation step. Include heteroduplex formation step in HRM protocol. Perform HRM reactions in white plastic plate.
Non-uniform fluorescence intensity	**Poor calibration of the thermal cycler.** Perform calibration of the real-time instrument according to the supplier's instructions. **Contamination of the thermal cycler.** Perform decontamination of your real-time instrument according to the supplier's instructions.

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- 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.
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