Manufactured by:
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QUANTIMIX HOTSPLIT PROBES KIT

Kit for real time DNA amplification and quantification using fluorescent-labeled specific probes

(Includes Biotools HotSplit DNA Polymerase)

Ref.	FORMAT	CONTENT
10.680M	20 rxn	Quantimix Hotsplit Probes Kit
10.681	100 rxn	Quantimix Hotsplit Probes Kit
10.682	200 rxn	Quantimix Hotsplit Probes Kit
10.683	500 rxn	Quantimix Hotsplit Probes Kit
10.685M	20 rxn	Quantimix Hotsplit Probes-ROX Kit
10.686	100 rxn	Quantimix Hotsplit Probes-ROX Kit
10.687	200 rxn	Quantimix Hotsplit Probes-ROX Kit
10.688	500 rxn	Quantimix Hotsplit Probes-ROX Kit
10.630M	20rxn	10X Quantimix Hotsplit Probes Kit
10.633	500 rxn	10X Quantimix Hotsplit Probes Kit

Store at -20ºC

Warning for users: Some of the applications that can be performed with this product are protected by patents applicable in some countries. Purchase of this product does not include or provide a licence to perform patented applications. In some cases, depending on the country and/or application, users are required to purchase a licence.

Ed 05 –Sept. 2021

1. PRODUCT DESCRIPTION

QUANTIMIX HOTSPLIT PROBES KIT is a master mix optimized for real-time amplification tests using fluorescent hydrolysis probes for the detection and quantification of template DNA. This kit is compatible with hydrolysis probes (eg Taqman®, ZEN™ double-quenched probes).

QUANTIMIX HotSplit Probes kit is available as Master Mix 2X format including all the elements necessary for DNA amplification, except template, primers and probe. The master mix format facilitates handling by reducing the operating time and pipetting steps.

The Quantimix HotSplit Probes composition includes the Biotools HotSplit DNA Polymerase, "hot start" enzyme ensuring optimum yield reaction by providing high specificity and sensitivity to amplification reactions, while minimizing spurious amplifications.

Some variants of the kit are provided with a Passive Reference Dye, ROX™, in order to normalise well-to-well differences that may occur due to artifacts such as pipetting errors or instrument (common to block-based thermocyclers). Reference Dyes are specially formulated for use on Applied Biosystems (ABI) and Stratagene real time PCR instruments. As the addition of ROX™ is optional, this reagent is provided in a separate tube.

2. REAGENTS INCLUDED IN THE KIT

- QUANTIMIX HotSplit Probes Mix: 2X or 10X master mix ready to use containing all the elements necessary for the real-time amplification: Biotools HotSplit DNA Polymerase, dNTPs, Reaction Buffer, stabilizers and $MgCl_2$

- ROX™ DYE: *Available for some references. It is supplied at 50X* concentration.

3. HANDLING AND STORAGE

Store QUANTIMIX HotSplit Probes Kit in a freezer at **-20 °C** to ensure a constant temperature (frost-free freezers are not recommended) and prevent frequent freeze/thaw cycles. For short term storage or frequent use, aliquots may be stored at 4 ° C.

ROX™ DYE should be stored in the dark at either -20ºC or 4ºC. Storage at 4ºC avoids the need of thawing the solution before assembling the PCR.

If the kit is handled and preserved following these recommendations, stability will be as indicated on the label.

Once the kit has been thawed, gently mix before use to minimize foaming. Keep the mixture on ice during handling.

4. INSTRUCTIONS

Template: Template DNA completeness and purity are key to obtain optimal results in real-time amplification reaction. While the DNA purified by standard methods is often adequate for amplification, components such as ionic detergents, dyes, phenol/chloroform, salts, EDTA and other chemical solvents may inhibit the amplification reaction and interfere with the detection of the fluorescent signal. It is recommended that DNA samples are transported cold to avoid deterioration.

Use between 10-1000 copies of template DNA for each amplification reaction; this amount of template is equivalent to approximately 100 pg-1 µg of gDNA or cDNA coming from total RNA 1 pg-100 ng. Excessive template can drag high levels of contaminants that adversely affect the efficiency of the reaction.

When using silica matrix methods for template DNA purification, make sure the silica particles in the sample have been completely removed, because they inhibit amplification and fluorescence reading. Biotools recommends its Speedtools line of products for the DNA extraction and purification from samples of different origin: blood (Speedtools DNA Extraction Kit), tissue (Speedtools Tissue DNA Extraction Kit), food (Speedtools Food DNA Extraction Kit) and plants (Speedtools Plant DNA Extraction Kit).

The amount of DNA to be added in the reaction depends on the origin and quality of the template to be included. Because of the qPCR high sensitivity, few copies of template DNA are usually sufficient to initiate amplification. To minimize the occurrence of inhibitors in the sample, you are recommended to start optimizing with the minimum amount of template required for efficient quantification. The kit has been optimized with different templates including: genomic DNA, cDNA and plasmid DNA.

ROX™ Concentration: Optimal concentration of Reference Dyes varies with the instruments. Generally, instruments with optimised filter sets require less concentration. Biotools recommends 1X of the provided solution. See specific instrument instructions for further details on passive dye usage.

Primer Design: The correct design of the primers is crucial for the success of the qPCR. The primers used in the amplification reaction usually have a size of 15 to 30 nucleotides with G+C levels of 40-60%. To avoid the primer-dimer or hairpin dimer formation, primers should not be complementary with themselves or with other primers present in the reaction.

When designing primers, note that the amplicons for real-time PCR should be 500 bp, with an optimum size between 75 and150 bp. To reduce the cycling time, selecting primers with a Tm close to 60°C and using 2-step amplification programs, instead of 3-step programs, is advisable.

The recommended primer concentration is 0.2-0.9 µM; use between 0.3-0.5 µM for initial optimization.

Regarding the probe concentration, although it depends on the type of probe used, the optimal concentration usually ranges between 0.1-0.5 µM; use 0.2 µM as basis concentration for optimization.

PCR program: Some programming parameters affect both amplification's specificity and efficiency.

The annealing temperature of the primers should be similar, with up to 5 °C difference between them. A low annealing temperature increases the risk of primer-dimers and/or nonspecific amplification products. For primers with Tm close to 60 °C, use 2-step amplification programs.

QUANTIMIX HotSplit Probes kit contains a chemically modified polymerase with little or no activity at low temperatures. The enzyme activity is recovered during the initial denaturation step. Apply between 5-10 min at 94 °C.

While Quantimix HotSplit Probes kit correctly amplifies by using reaction volumes between **20-50 µL**, recommended reaction volumes are 20 to 25 µL.

5. STANDARD PROTOCOL

Materials to be provided by the user:

- *Specific fluorescent probe*
- *Specific primers*
- *nuclease-free water*
- *Template DNA, positive control for the standard curve*

Laboratory workflow should be unidirectional, from pre-amplification area to amplification area. To avoid cross-contamination using specific equipment for each work area is recommended.

KEEP THE REACTION TUBES COOL until being inserted in the thermocycler. When working with standard vials, be sure to keep them on ice or in cooled blocks preventing the covers from getting wet.

Work in the reagent preparation area, in a laminar flow cabinet. To avoid contamination or false negatives, use gloves and nuclease-free plastic material, and pipette tips with filter.

1.-Thaw and mix Quantimix HotSplit Probes mix gently before dispensing.

2.- If the test is quantitative, standards of known concentration should be included for performing a standard curve. You are recommended to include at least one negative and one positive control for each experiment performed.

3.- Prepare the reaction mixture following the instructions of Table 1. AVOID MXTURE PROLONGED EXPOSURE TO LIGHT.

4.-When required include Passive Reference Dye (ROX™) into the master mix. Keep all tubes containing ROX™ protected from light.

TABLE 1. Preparation of the reaction mixture

COMPONENT	Final Concentration	20 µL rxn	25 µL rxn	50 µL rxn
2X Quantimix HotSplit Probes Mix	1 X	10 µL	12.5 µL	25 µL
Primers	0.2-0.9 µM	x µL	x µL	x µL
Probe	0.1-0.5 µM	x µL	x µL	x µL
ROX™ Dye	1X	0,4 µl	0,5 µl	1 µl
Nuclease free water	-	Up to 20 µL	Up to 25 µL	Up to 50 µL
Template DNA	variable	x µL	x µL	x µL

COMPONENT	Final Concentration	20 µL rxn
10X Quantimix HotSplit Probes Mix	1 X	2 µL
Primers	0.2-0.9 µM	x µL
Probe	0.1-0.5 µM	x µL
Nuclease free water	-	Up to 20 µL
Template DNA	variable	x µL

Proceed to work in the area of DNA purification away from other sources of DNA.

Do not insert the DNA in the laminar flow cabinet from the reagent preparation area. It is desirable that the amplification begin no later than 10 minutes after the DNA and primers have been added to the reaction mixture. Place all reagents on ice until their insertion in the thermocycler.

5.- Add the template DNA to each reaction vial. Close the vials and mix carefully (do not use vortex) and centrifuge briefly before starting cycling.

Proceed to the amplification area or PCR

6.- Program the thermocycler. In Table 2, a 3-step and a 2-step program are recommended in accordance with the Tm of the primers. The recommended cycling parameters are only a guideline and they may be changed depending on the characteristics of each test.

TABLE 2. PCR Program for QUANTIMIX HotSplit Probes kit

A) 3-Step Amplification Program (for primers with T_m< 55°C)

STEPS	N° of cycles	Temperature	Time
Initial denaturation	1	94ºC	8 min
Denaturation	30-45	94-96ºC	10-30 sec
Annealing		2-5 ºC □Tm of primers	10-30 sec
Extension*		60-67ºC	60 sec

*** Reading fluorescence in step Extension**

B) 2-Step Amplification Program (for primers with $T_m \geq$ 55°C)

STEPS	N° of cycles	Temperature	Time
Initial denaturation	1	94ºC	8 min
Denaturation	30-45	94-96ºC	10-30 sec
Annealing/Extension		60ºC	60 sec

*** Reading fluorescence in step Annealing/Extension**

***Note:* When using ROX™ Dye fluorescence acquisition for specific fluorophore and ROX™ is done simultaneously at the appropriate channel.**

7.- Place the tubes in the thermocycler and start the cycling.

The results will be interpreted with the specific software following the supplier's instructions and recommendations.

6. TROUBLESHOOTING

Lack of amplification or low reaction efficiency

1. **Verify the DNA quality and quantity.** Verify the template quality and quantity by agarose gel electrophoresis or fluorimetry. If necessary, perform an organic extraction followed by ethanol precipitation to remove inhibitors. Excessive template can diminish reaction yield.
2. **Improper storage of primers and probe.** The deterioration of primers and probes caused by improper storage diminishes the specificity of the reaction and the signal to noise ratio (fluorescence).
3. **Presence of inhibitors in the reaction mixture.** Verify the presence of inhibitors by performing serial dilutions of the template DNA. If the reaction yield is higher (lower ct) in higher dilutions, the presence of inhibitors will be seen and the reaction should be repeated with a new sample of conveniently purified DNA.
4. **Inefficient reverse transcription** When cDNA is used as template, low reaction efficiency may be due to a failure in the reverse transcription reaction. Verify that the conditions of this reaction are the appropriate and that the template obtained is not deteriorated.
5. **Lack of uniformity in reagents** Verify that the reaction reagents have been mixed correctly before placing the vials in the thermocycler.
6. **Problem with primers.** Designing primers with a higher annealing temperature without causing hairpins and/or primer dimers.
   Optimise the primer concentration: A poor concentration of primers will diminish the amplification reaction yield. Increase the primer concentration in 0.1 µM.
7. **Reduce the size of the PCR product.** Design new primers to obtain PCR product sizes between 75-150 bp.
8. **Verify that the fluorescent probe is specific for the template to be amplified.** Also check the concentration of the probe included in the reaction.
9. **Increase the time of initial denaturation.** Extend the time of initial denaturation, especially if your template has secondary structures or high G+C levels (up to 11 min).
10. **Decrease annealing temperature.** Choose the 3-step amplification program and decrease annealing temperature in decrements of 2 °C.
11. **Increase number of cycles.** Add more cycles in the amplification program in increments of 5 cycles.
12. **Verify detection step program.** Verify that the fluorescence detection step has been activated and that it takes place at the appropriate step of the amplification program.
13. **Select an appropriate filter.** For real-time thermocyclers having multiple detection channels, verify that the activated filter is consistent with the probe labeling.

Nonspecific amplification products

1. **Reduce the concentration of the reaction components.** Verify the template DNA concentration by agarose gel electrophoresis or fluorimetry. Reduce the amount of template, primers and/or probe in the reaction mixture.
2. **Verify the status of the primers.** Verify that the primers are not deteriorated by performing a denaturing acrylamide gel electrophoresis.
3. **Redesign the primers.** Design primers with a higher Tm without causing hairpins and/or primer dimers.
4. **Problem with probe.** Verify that the probe is specific for the template to be amplified. Optimize probe concentration, reduce the amount of probe in decrements of 0.05 µM.
5. **Increase annealing temperature.** Use higher annealing temperatures, in increments of 2 °C or chose a 2-step amplification program.
6. **Reduce number of cycles.** Reduce the number of cycles of the amplification program in increments of 5 cycles.