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QUANTIMIX HOTSPLIT EASY KIT

Kit for Real Time DNA Amplification and Quantification to use with Intercalating Fluorophores

(Includes Biotools HotSplit DNA Polymerase)

Ref.	FORMAT	CONTENT
10.690M	20 rxn	Quantimix Hotsplit Easy Kit
10.691	100 rxn	Quantimix Hotsplit Easy Kit
10.692	200 rxn	Quantimix Hotsplit Easy Kit
10.693	500 rxn	Quantimix Hotsplit Easy Kit
10.695M	20 rxn	Quantimix Hotsplit Easy-ROX Kit
10.696	100 rxn	Quantimix Hotsplit Easy-ROX Kit
10.697	200 rxn	Quantimix Hotsplit Easy-ROX Kit
10.698	500 rxn	Quantimix Hotsplit Easy-ROX Kit

Store at -20ºC

Research Use Only. Not for use in diagnosis procedures

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1. DESCRIPTION

The **QUANTIMIX HOTSPLIT EASY KIT** is a universal master mix optimised to deliver maximum efficiency, precision, and sensitivity during nucleic acid amplification in real time using SYBR® Green I. The mixture includes a chemically modified version of Biotools DNA Polymerase, Biotools HotSplit DNA Polymerase, which presents little or no activity at low temperatures and provides greater specificity and yield in DNA amplifications.

Real time detection of amplified products is performed by monitoring the increase of fluorescence after each amplification cycle. In QUANTIMIX HOTSPLIT EASY Kit the fluorescent signal is generated by the incorporation of SYBR® Green I to the amplified products. In the exponential phase of the amplification there is a correlation between the amount of product and the initial template DNA and the amount of fluorescence is proportional to the amplified DNA in each cycle.

The Biotools HotSplit DNA Polymerase ensures high specificity and sensitivity. It overcomes the problems due to nonspecific priming, primer-dimer formation or unwanted reactions occurring during the PCR setting. The presence of thermolabil blocking groups acting over the amino-acid residues involved in enzyme polymerization; the polymerase activity is restored during the initial denaturation step. On the other hand, Biotools HotSplit DNA Polymerase has a low base misincorporation rate in comparison with similar commercial enzymes.

Some variants of the kit are provided with a Passive Reference Dye, ROX^{TM}, 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^{TM} is optional, this reagent is provided in a separate tube.

QUANTIMIX HOTSPLIT EASY KIT is a ready-to-use 2X Master Mix (QUANTISPLIT), which includes all reaction components except template and primers.

2. REAGENTS INCLUDED IN THE KIT

- ***QUANTISPLIT:*** It is a 2X ready-to-use solution that contains all necessary components for real time amplification assays: Biotools HotSplit DNA Polymerase, all four dNTPs, Reaction Buffer, SYBR® Green I and $MgCl_2$ is also included at the appropriate concentration (4mM).
- ***50 mM $MgCl_2$ Solution:*** Used only for specific real time assays which require an additional optimisation.
- ***ROX^{TM} DYE:*** Available for some references. It is supplied at 50X concentration.

3. STORAGE AND HANDLING INSTRUCTIONS

Store all components of the QUANTIMIX HOTSPLIT EASY Kit at **-20ºC** in a constant temperature freezer (frost-free freezers are not recommended). All reagents must be thawed and handled on ice.

QUANTISPLIT: It is a light-sensitive solution; therefore the vial should be protected from light whenever possible. Minimise the number of freeze-thaw cycles by storing in working aliquots. Mix before use.

$MgCl_2$ Solution: Mix thoroughly before use.

If stored under the recommended conditions, the product will maintain performance through the indicated date on the label.

4. GENERAL CONSIDERATIONS

Template: The quality of the DNA template is key to obtaining optimal results in amplification reactions. Although conventional extraction methods produce templates with sufficient quality for PCR, some of the reagents used during purification (phenol, EDTA, proteinase K, ionic detergents, silica particles, etc.) often inhibit amplification. Biotools recommends its Speedtools product range for the extraction and purification of genomic DNA from blood (*Speedtools DNA Extraction*); tissue (*Speedtools Tissue DNA Extraction kit*); food (*Speedtools Food DNA Extraction kit*); and from plants (*Speedtools Plant DNA Extraction kit*).

Samples should be transported cold as a lack of refrigeration may lead to degradation of the DNA. All clinical samples must be handled as though they were potentially infectious.

The quantity of template DNA to be added in each reaction depends on the source and quality of the template. We recommend determining the concentration by fluorimetry. If you do not know the concentration of template DNA, add a fixed volume of the extraction mixture to the problem samples. The purpose of this recommendation is to obtain comparable quantitative results.

$MgCl_2$ Concentration: Magnesium ion concentration affects primer annealing and template denaturation, as well as enzyme activity and fidelity. Generally, excess Mg^{2+} results in accumulation of nonspecific amplification products, whereas insufficient Mg^{2+} results in reduced yield of the desired PCR product. The recommended range of concentration is 4-6 mM. A 4mM total $MgCl_2$ concentration is present in the final 1X dilution of the QUANTISPLIT (appropriate concentration). However, the kit is provided with an additional vial with 50 mM $MgCl_2$ Solution for additional optimisation.

ROX^{TM} 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: Primers typically are 15-30 bases in length and contain approximately 40-60% G+C residues: the annealing temperatures of primer pairs should be nearly identical. Care must be taken to design primers that do not form hairpin loop structures or are self-complementary. The 5´ end of a primer may contain mismatches between the primer and template, whereas this is not recommended at the 3´ end.

For qPCR with SYBR® Green I, relatively low primer concentrations are used to avoid primer-dimer formation. For most SYBR® Green I applications, primer concentrations ranging from 0.05-0.3 µM should be tested.

Unlike regular PCR, amplicons for real time PCR tend to be less than 500 bp. The optimal real-time PCR results can be achieved with amplicon sizes of 100-300 bp; longer products do not amplify as efficiently.

Cycling Parameters: Many parameters influence both the specificity and efficiency of amplification including the temperature and duration of denaturation, annealing and elongation; ramp speed; and total cycle number. Due to the shorter length of real time PCR amplicons, cycling programs for real time PCR are typically shorter than regular PCR. Variations to the program may be required depending on the amplicon size.

QUANTIMIX HOTSPLIT EASY Kit contains a chemically modified polymerase which presents little or no activity at low temperature. The full enzyme activity is recovered during the initial denaturation step; 10 min at 96°C should be enough to completely activate the polymerase.

Biotools HotSplit DNA Polymerase also has a low base misincorporation rate and requires more time for extension at 72ºC as compared to other polymerases.

The annealing temperature should be 5ºC lower than the theoretical melting temperature of the primers. If the annealing temperature is set too low, there is an added risk of primer-dimer extension or nonspecific products.

A ***dissociation or melting curve*** is essential when running assays using intercalating fluorophores in order to check the melting profile of PCR products.

5. STANDARD PROTOCOL

Materials to be supplied by user:

- Downstream oligonucleotide primer
- Upstream oligonucleotide primer
- Nuclease-free water

Laboratory workflow must be unidirectional, from pre-amplification to amplification areas. Specific equipment for each working area must be used, in order to avoid cross contaminations.

For optimal results, it is essential to KEEP THE REACTION VIALS REFRIGERATED until their introduction in the thermal cycler.

Proceed to the Reagent Preparation Area in a laminar flow cabinet. Wear disposable gloves and use sterile and nuclease free plastic material in order to avoid contaminations and false negative results. We also recommend the use of filter tips.

1.-Thaw and thoroughly mix all the reagents before dispensing (avoid vortex). Protect amplification vials containing Quantisplit master mix from light at all times.

2.-Determine the number of samples to be analysed. If quantification is performed include samples of known concentrations that will be used for the standard curve. The inclusion of positive and negative controls is highly recommended in each experiment.

3.-Prepare the reactions according to Table 1. PROTECT MASTER MIX FROM 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. Master Mix preparation

COMPONENT	Final Concentration	20 µl rxn
2X QUANTISPLIT	1 X	10 µl
50 mM $MgCl_2$ Solution*	4-6 mM	x µl
Primers	0.05-03 µM	x µl
ROX™ Dye	1X	0,4 µl
Sterile bidistilled water	-	Up to 20 µl
DNA template	variable	x µl

*Only necessary for concentrations of $MgCl_2$ >4mM

Proceed to DNA Purification Area separate from other sources of DNA.

Never introduce DNA in the laminar flow cabinet from the reagent preparation area. Amplification must start in the next 10 minutes after adding purified DNA and primers to the amplification mix. Keep all reagents on ice until their introduction in the thermal cycler.

5.-Add template DNA to each reaction tube. Close the tubes and mix gently.

6.-Centrifuge the amplification vials briefly.

Proceed to Amplification Area

7.-Place the tubes in the thermal cycler and begin cycling.

8.-Program the thermal cycler following the recommendations provided by the manufacturer (see TABLE 2).

TABLE 2. PCR cycling parameters for QUANTIMIX HOTSPLIT EASY Kit

Cycle Step	**Nº Cycles**	**Temperature**	**Time**
Initial Denaturation and **Enzyme Activation**	1	94-96ºC	8-10 min
Denaturation	30-50	95-98ºC	5-20 sec
Annealing		2-5°C<Tm of the primers	5-10 sec
Extension*		70-72ºC	30-60 sec
Fluorescence Acquisition **(See Note 1)**	1	Tm of nonspecific products <X<Tm specific products	10-15 sec+
Melting*	1	60-95ºC	Start with a 0.5 ºC/ sec ramp

* Fluorescence Acquisition during Extension and Melting Steps.
+ The shortest time needed for reading fluorescence (different for each thermal cycler).

Note 1: **When primer-dimer or nonspecific products appear, include an additional Fluorescence Acquisition Step after the Extension Step**
Note 2: **When using ROX™ Dye, fluorescence acquisition for specific fluorophore and ROX™ is done simultaneously at the appropriate channel.**

The interpretation of the results is performed with the help of specific software. Therefore, follow the instructions and advice provided by the manufacturer.

6. TROUBLESHOOTING

Little or no amplification detected

1. **Check template quality and quantity.** Check the quality of template DNA by agarose gel electrophoresis or fluorimetry. Organic extraction followed by ethanol precipitation may remove some amplification inhibitors. Use of excess template can reduce PCR product yield.
2. **Redesign primers.** Design primers that have higher annealing temperatures and do not form hairpin loops or primer-dimers.
3. **Optimise primer concentration.** Although lower primer concentration can prevent primer-dimer formation, sufficient primers are needed for successful real-time PCR. Increase primer concentration in increments of 0.1 µM.
4. **Increase initial template denaturation time.** Increase the length of initial template denaturation up to 10 minutes in order to ensure that HotSplit DNA Polymerase has been completely activated.
5. **Lower annealing temperature.** Lower the annealing temperature in 2°C decrements.
6. **Increase number of cycles.** Perform additional cycles in increments of 5 cycles.
7. **Increase extension time.** Increase the extension time by increments of 30 sec. Generally 20 seconds/100 bp of PCR product should be enough.
8. **Check detection step accuracy.** Ensure the fluorescence detection step takes place during the correct step of the PCR cycling program.
9. **Choose a filter compatible with your dye.** For a real-time instrument that is equipped with a multiple dye detection system, ensure that a SYBR® Green I dye-compatible filter is activated.

Multiple products or a smear detected

1. **Decrease concentration of reaction components.** Check the concentration of template DNA by agarose gel electrophoresis or fluorimetry. Decrease the amount of DNA and/or primer added to the reaction.
2. **Check the primers for degradation.** Check by electrophoresis in a denaturing acrylamide gel.
3. **Decrease primers concentration.** For SYBR® Green I, relatively low primer concentrations are used to avoid primer-dimer formation (0.05-0.3 µM).
4. **Redesign primers.** Design primers that have higher annealing temperatures and do not form hairpin loops or primer dimers.
5. **Increase annealing temperature.** Increase the annealing temperature in 2°C increments.
6. **Decrease number of cycles.** Decrease number of cycles in decrements of 5 cycles.
7. **Decrease PCR product size.** For optimal real time PCR, design primers that amplify PCR products between 100-300 bp in length.
8. **Post-amplification Melting Curve.** Since SYBR® Green I is a DNA binding dye, it will generate signal from both specific and non-specific products. The generation of all products can be easily visualised on a melt curve following the amplification reaction.

No linearity in amplification curve

1. **Check template quantity.** An excess of template can affect the linearity.
2. **Include an additional Fluorescence Acquisition Step.** Non specific amplification products can affect the linearity (See Note 1).
3. **Post-amplification Melting Curve.** It could be essential to check the melting profile of PCR products.

Fluorescence in no template control (NTC)

1. **Repeat the assay with new reagents and take appropriate safety precautions.** If the Tm of the NTC is similar to the Tm of the target (contamination).
2. **Include an additional Fluorescence Acquisition Step.** If Tm of NTC is lower than the Tm of the target (primer-dimers) (See Note 1).