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3B QUANTIMIX EASY PROBES KIT

PRODUCT	FORMAT	REF.
Quantimix Easy Probes kit	100 rxn	3B106
Quantimix Easy Probes kit	200 rxn	3B107
Quantimix Easy Probes kit	500 rxn	3B108
Quantimix Easy Probes kit	1000 rxn	3B109

1. DESCRIPTION

3B QUANTIMIX EASY PROBES KIT has been optimised to deliver maximum efficiency, precision, and sensitivity during nucleic acid amplification in real time using fluorescent target-specific probe(s) for amplicon detection and quantification. The kit is compatible with hydrolysis probes (e.g. Taqman®) and hairpin probes (e.g. Scorpion®).

Real time detection of amplified products is performed by monitoring the increase of fluorescence after each amplification cycle. In QUANTIMIX EASY PROBES Kit the fluorescent signal is generated by the use of reporter molecules that can be part of sequence specific probe(s). 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.

QUANTIMIX EASY PROBES KIT is a 2X Master Mix (QUANTIPROBES), which contains: 3B DNA polymerase, all four dNTPs, $MgCl_2$ and Reaction Buffer. It is a convenient ready-to-use Master Mix that reduces set up time and liquid handling steps. All reaction components are included in the QUANTIPROBES, except template, primers and fluorescent probe(s).

The kit is compatible with real-time PCR thermal cyclers that use either standard or capillary reaction vials.

2. REAGENTS INCLUDED IN THE KIT

- ***QUANTIPROBES:*** It is a ready-to-use 2X solution that contains all necessary components for real time amplification assays: 3B DNA Polymerase, dNTPs, Reaction Buffer and $MgCl_2$ is also included at the appropriate concentration (final concentration: 4mM).
- ***50 mM $MgCl_2$ Solution:*** Used only for specific real time assays which require an additional optimisation.

3. STORAGE AND HANDLING INSTRUCTIONS

Store all components of the QUANTIMIX EASY PROBES Kit at **-20ºC** in a constant temperature freezer (frost-free freezers are not recommended). All reagents must be thawed and handled on ice. For frequent use, divide in aliquots to avoid multiple freeze-thaw cycles.

- **QUANTIPROBES:** Mix thoroughly 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: DNA condition is a key point to obtain optimal results. DNA prepared using standard isolation techniques is a suitable substrate for amplification. Nevertheless, numerous compounds inhibit amplification including ionic detergents, some gel loading dyes, phenol and hemin.

The employed methods for DNA purification can be either phenol-based or resin-based, provided enough amount of pure DNA and guarantee the absence of amplification inhibitors. When using purification methods based on a silica matrix, it is important to check the complete absence of silica particles in the samples since it inhibits amplification and fluorescence reading. Samples should be transported and stored frozen. In samples that have been stored without refrigeration, DNA can be degraded. When you work with clinical samples, handle them as if they are capable of transmitting infectious agents.

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 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 $MgCl_2$ concentration is present in the final 1X dilution of the QUANTIPROBES (optimal concentration). However, the kit is provided with an additional vial with 50 mM $MgCl_2$ for additional optimization.

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.

In order to shorten cycling times, chose primers with a Tm close to 60 °C, so a 2-step cycling program can be used. The recommended primer concentration is 0.3-1.0 µM.

Unlike regular PCR, amplicons for real time PCR tend to be less than 500 bp. Optimal real-time PCR results can be achieved with amplicon of 75-150 bp.

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.

As QUANTIMIX EASY PROBES Kit contains an enzyme with a low base misincorporation rate (1-10 x 10^{-6} bp) it requires more time for extension as compared to other polymerases.

The annealing temperature should be 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.

5. STANDARD PROTOCOL

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

KEEP THE REACTION VIALS REFRIGERATED until their introduction in the thermal cycler. When working with standard conical amplification vials, be sure to keep them on ice or in coolers, avoiding wetting the optical cap. If capillary vials are employed, make sure that the cooler has been at 4ºC at least for 4 hours before use.

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.

2.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.

TABLE 1. Master Mix preparation

COMPONENT	Final Concentration	20 µl rxn
2X QUANTIPROBES	1 X	10 µl
50 mM $MgCl_2$ Solution*	4-6 mM	x µl
Primers	0.3-1 µM	x µl
Probe(s)	0.3-1 µM	x µl
BSA[1]	0.5 mg/ml	x µl
Sterile bidistilled water	-	Up to 20 µl
DNA template	variable	x µl

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

[*] we recommends the addition of BSA if real time assays are to be carried out in the glass capillary tubes of the Roche LightCycler

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 your thermal cycler following the recommendations provided by the manufacturer. A 2-step or 3-step suggested program is outlined on TABLE 2. We recommended a 2-step cycling program for primers with a Tm of 60°C of higher.

TABLE 2. PCR cycling parameters for QUANTIMIX EASY PROBES Kit

A) 3-Step Protocol (for primers with a T_m less than 60°C)

Cycle Step	Nº Cycles	Temperature	Time
Initial Denaturation	1	95-98ºC	2-5 min
Denaturation	30-50	95-98ºC	5-10 sec
Annealing		Tm of the primers	5-10 sec
Extension*		60-65ºC	20-60 sec(20 sec/100 bp)

***Fluorescence acquisition during the Extension Step**

B) 2-Step Protocol (for primers with a Tm of 60°C or higher)

Cycle Step	Nº Cycles	Temperatu re	Time
Initial Denaturation	1	95-98ºC	2-5 min
Denaturation	30-50	95-98ºC	5-10 sec
Annealing/Extension*		Tm of the primers	20-60 sec (20sec/100 bp)

***Fluorescence acquisition during the Annealing/Extension Step**

This protocol has been optimised for the following real time quantification equipments: LightCycler (Roche), iCycler (Biorad), SmartCycler I and II (Cepheid), Rotor-Gene 3000 and 6000 (Corbett Research) and ABI PRISM 7500 series (Applied Biosystems). For other thermal cyclers, optimisation of the reaction parameters may be required. Please contact our Technical Department.

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 dimmers.
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. **Ensure that the fluorescent-labeled probe is designed specifically for the target template.**
5. **Optimise probes concentration.** Check reaction conditions of your specific fluorescent-labelled probe(s).
6. **Increase initial template denaturation time.** Increase the length of initial template denaturation up to 5 minutes. Templates rich in G+C content or with secondary structures often require longer initial denaturation.
7. **Lower annealing temperature.** Lower the annealing temperature in 2°C decrements.
8. **Increase number of cycles.** Perform additional cycles in increments of 5.
9. **Increase extension time.** Increase the extension time by increments of 30 sec. Usually 20 seconds/100 bp of PCR product should be enough.
10. **Check detection step accuracy.** Ensure the fluorescence detection step takes place during the correct step of the PCR cycling program.
11. **Choose the appropriate filter.** For a real-time instrument that is equipped with a multiple dye detection system, ensure that the appropriate filters are 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. **Redesign primers.** Design primers that have higher annealing temperatures and do not form hairpin loops or primer-dimers.
4. **Ensure that the fluorescent-labeled probe is designed specifically for the target template.**
5. **Optimise probes concentration.** Check reaction conditions of your specific fluorescent-labeled probe(s)
6. **Increase annealing temperature.** Increase the annealing temperature in 2°C increments.
7. **Decrease number of cycles.** Decrease number of cycles in decrements of 5 cycles.

Decrease PCR product size. For optimal real time PCR, design primers that amplify PCR products between 75-150 bp in length.