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# QUANTIMIX EASY PROBES KIT

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

| Ref. | FORMAT | CONTENT |
|---|---|---|
| 10.600M | 20 rxn | Quantimix Easy Probes Kit |
| 10.601 | 100 rxn | Quantimix Easy Probes Kit |
| 10.602 | 200 rxn | Quantimix Easy Probes Kit |
| 10.603 | 500 rxn | Quantimix Easy Probes Kit |
| 10.604 | 1000 rxn | Quantimix Easy Probes Kit |
| 10.650M | 20 rxn | Quantimix Easy Probes-ROX Kit |
| 10.651 | 100 rxn | Quantimix Easy Probes-ROX Kit |
| 10.652 | 200 rxn | Quantimix Easy Probes-ROX Kit |
| 10.653 | 500 rxn | Quantimix Easy Probes-ROX Kit |
| 10.654 | 1000 rxn | Quantimix Easy Probes-ROX Kit |

**Store at -20ºC**

***Research Use Only. Not for use in diagnosis procedures***

***Notice to users:*** Some of the applications which may be performed with this product are covered by applicable patents in certain countries. The purchase of this product does not include or provide a license to perform patented applications. Users may be required to obtain a license depending on the country and/or application.

Ed .08 – March 2017

## 1. DESCRIPTION

**QUANTIMIX EASY PROBES KIT** has been optimised to deliver maximum efficiency 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®; ZEN™ *double-quenched probes*) 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 probes. 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.

Some references 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 EASY PROBES KIT** is a 2X Master Mix (QUANTIPROBES), which contains: Biotools 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.

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: Biotools 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.
- ***ROX$^{TM}$ DYE:*** Available for some references. It is supplied at 50X concentration.

## 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.
- **ROX$^{TM}$ DYE:** Should be **stored in the dark** at either **-20ºC** or **4°C.** Storage at 4°C avoids the need of thawing the solution before assembling the PCR.

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

## 4. GENERAL CONSIDERATIONS

***Template:*** Integrity and purity of DNA template is essential to obtain optimal results in qPCR. 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/chloroform, salts, EDTA and other chemical solvents. Contaminants can also interfere with fluorescence detection. Samples should be transported and stored frozen. In samples that have been stored without refrigeration, DNA can be degraded.

When using DNA 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. We recommend the use of our Speedtools line for extraction of DNA from blood (*Speedtools DNA Extraction Kit*); from tissue (*Speedtools Tissue DNA Extraction Kit*); from food (*Speedtools Food DNA Extraction Kit);* and from plant material (*Speedtools Plant DNA Extraction Kit*).

The quantity of template DNA to be added in each reaction depends on the source and quality of the template. If you do not know the concentration of template DNA, add a fixed volume of the extraction mixture to the problem samples. Very few copies of target DNA are needed to initiate qPCR. To minimise contamination with reaction inhibitors, the starting template amount should be kept to minimum required to achieve accurate quantification.

***$MgCl_2$ 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 3-6 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 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:*** Whether using an intercalating dye or a probe-based detection, designing high-quality primers is one of the most crucial pre-experimental steps in qPCR. 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 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 0.1-0.9 μM.

Unlike regular PCR, amplicons for real time PCR tend to be less than 500 bp; optimal qPCR results can be achieved with amplicon of 50-150 bp.

Regarding ***probes concentration***, although it depends on the probes, the recommended concentration is 0.1-0.5 μM (0.2 μM should be the optimal).

***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.

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.

As QUANTIMIX EASY PROBES Kit contains an enzyme with a low base misincorporation rate it requires more time for extension as compared to other polymerases.

## 5. STANDARD PROTOCOL

***Materials to be supplied by user:***

- Fluorescent-labeled specific probe
- qPCR primers
- Nuclease-free water
- DNA template, positive-control template standards

***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.

4.-When required include Passive Reference Dye (ROX$^{TM}$) into the master mix. Keep all tubes containing ROX$^{TM}$ protected from 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 | 0.1-0.9 µM | x µl |
| Probe | 0.1-0.3 µM | x µl |
| ROX$^{TM}$ Dye | 1X | 0,4 µl |
| BSA[1] | 0.5 mg/ml | x µl |
| Nuclease free-water | - | Up to 20 µl |
| DNA template | variable | x µl |

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

[1]Biotools 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 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, mix gently and centrifuge them briefly.

***Proceed to Amplification Area***

6.-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. The cycling parameters given bellow are offered as a guideline and may be modified as necessary.

***TABLE 2. PCR cycling parameters for QUANTIMIX EASY PROBES Kit***

**A) 3-Step Protocol (for primers with a $T_m$ □ 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 | | 2-5°C<Tm of 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 ≥ 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/Extension* | | 60ºC | 60 sec |

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

***Note*: When using ROX$^{TM}$ Dye, fluorescence acquisition for specific fluorophore and ROX$^{TM}$ is done simultaneously at the appropriate channel**

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

*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 (info@biotools.eu).*

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.**
1. **Optimise probes concentration.** Check reaction conditions of your specific fluorescent-labeled probes.
2. **Decrease PCR product size.** For optimal real time PCR, design primers that amplify PCR products between 75-150 bp in length.
5. **Increase initial template denaturation time.** Increase the length of initial template denaturation up to 7 minutes. Templates rich in G+C content or with secondary structures often require longer initial denaturation.
6. **Lower annealing temperature.** Lower the annealing temperature in 2°C decrements.
7. **Increase number of cycles.** Perform additional cycles in increments of 5 cycles.
8. **Increase extension time.** Increase the extension time by increments of 30 sec. Usually 20 seconds/100 bp of PCR product should be enough.
9. **Check detection step accuracy.** Ensure the fluorescence detection step takes place during the correct step of the PCR cycling program.
10. **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 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 probes.
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.