Produced by:
BIOTOOLS, Biotechnological & Medical Laboratories, S.A. have been evaluated and certified to accomplish ISO 9001:2008 requirements for the following activities: Research and development of biotechnology products and manufacture of biotechnology and in vitro products. Valle de Tobalina – 52 – Nave 39, 28021 Madrid – Spain

BIOTOOLS B&M Labs, S.A.
Valle de Tobalina - 52 - Nave 39
28021 Madrid
Spain

Tel. (34) 91 710 00 74
Fax (34) 91 505 31 18
E-mail: info@biotools.eu
www.biotools.eu

BIOTOOLS B&M LABS.S.A.

QUANTIMIX EASY KIT

Kit for Real Time DNA Amplification and Quantification to use with intercalating fluorophores

Ref.	FORMAT	CONTENT
10.607M	20 rxn	Quantimix Easy Kit
10.607	100 rxn	Quantimix Easy Kit
10.608	200 rxn	Quantimix Easy Kit
10.609	500 rxn	Quantimix Easy Kit
10.606	1000 rxn	Quantimix Easy Kit
10.657M	20 rxn	Quantimix Easy-ROX Kit
10.657	100 rxn	Quantimix Easy-ROX Kit
10.658	200 rxn	Quantimix Easy-ROX Kit
10.659	500 rxn	Quantimix Easy-ROX Kit
10.656	1000 rxn	Quantimix Easy-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 .09 – March 2017

1. DESCRIPTION

The **QUANTIMIX 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.

Real time detection of amplified products is performed by monitoring the increase of fluorescence after each amplification cycle. In QUANTIMIX 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.

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.

QUANTIMIX EASY KIT is a ready-to-use 2X Master Mix (QUANTIMIX EASY MASTER MIX), which contains: Biotools DNA polymerase; all four dNTPs; $MgCl_2$; reaction buffer; and SYBR® Green I. The easy-to-use Master Mix reduces set up time and liquid handling steps. All reaction components are included in the Master Mix, except template and primers.

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

2. REAGENTS INCLUDED IN THE KIT

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

3. STORAGE AND HANDLING INSTRUCTIONS

Store all components of the QUANTIMIX EASY 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.

- **QUANTIMIX EASY MASTER MIX:** It is a **light-sensitive** solution, therefore the vial should be **protected from light** whenever possible. Mix before use.
- **$MgCl_2$ Solution:** Mix thoroughly before use.
- **ROX™ DYE:** Should be **stored in the dark** at either **-20°C** 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: 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.

When using DNA purification methods based on a silica matrix, it is important to check the complete absence of silica particles in the sample since it inhibits amplification and fluorescence reading. We recommend the use of our Speedtools line for extraction of genomic DNA from blood (Speedtools DNA Extraction); from tissue (Speedtools Tissue DNA Extraction Kit); from food (Speedtools Food DNA Extraction Kit); and from plant material (Speedtools Plant DNA Extraction Kit).

Samples should be transported and stored frozen. In samples that have been stored without refrigeration, DNA can be degraded. In case of working 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 QUANTIMIX EASY MASTERMIX (optimal concentration). However, the kit is provided with a vial with 50 mM $MgCl_2$ for additional optimisation.

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: 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.

As QUANTIMIX EASY Kit contains an enzyme with a low base misincorporation rate (1-10 x 10^{-6} bp) it 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 primers. If the annealing temperature is set too low, there is an added risk of primer-dimer extension or nonspecific products.

The ***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.

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. Protect amplification vials containing Quantimix Easy Master Mix from light at all times.

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™) into the master mix. Keep all tubes containing ROX™ protected from light.

TABLE 1. Master Mix preparation

COMPONENT	Final Concentration	20 µl rxn
2X QUANTIMIX EASY MASTER MIX	1 X	10 µl
50 mM $MgCl_2$ Solution*	4-6 mM	x µl
Primers	0.05-0.3 µM	x µl
ROX™ Dye	1X	0,4 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
[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 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 EASY Kit

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 the primers	5-10 sec
Extension*		70-72ºC	30-60 sec (20 sec/100 bp)
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.**

This protocol has been optimised for the following real time quantification equipment: 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 your 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 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. Templates rich in G+C content or with secondary structures often require longer initial denaturation (≤ 7 min).
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. **Include an additional Fluorescence Acquisition Step.** See Note 1.
9. **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.