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QUANTIMIX LIONPROBES[®] KIT

Kit for DNA LIONPROBES[®]

Real Time Amplification and quantification

Package Insert

Ref. 10.611/10.612/10.613

**PLEASE READ THE INSTRUCTIONS FOR USE THOROUGHLY BEFORE USING THE KIT,
ESPECIALLY IF YOU ARE NOT FAMILIAR WITH THE PROTOCOL.**

QUANTIMIX LIONPROBES[®] KIT

**Research Use Only (RUO)
Not for use in diagnosis procedures**

Some of the applications which may be performed with this product may be in certain countries under an applicable patent. 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. Biotools does not encourage the unlicensed use of patented applications.

PLEASE CHECK INTEGRITY OF KIT AND REAGENTS BEFORE USE. DETERIORATED KITS MAY CAUSE EQUIVOCAL RESULTS.

1. INTENDED USE

QUANTIMIX LIONPROBES[®] Kit allows the preparation and optimization of nucleic acid amplification mixtures for Real Time amplification applications using specific probes and primers designed with LIONPROBES[®] technology

The kit can be used in a wide variety of Real Time amplification equipments, including air heating amplification systems (using capillary tubes) or block heating amplification systems (using Cepheid tubes or conical tubes).

The employed methods for DNA purification can be either phenol-based or resin-based, provided they yield enough amount of pure DNA and guaranteeing absence of amplification inhibitors. When using purification methods based on silica matrix, it is most important checking complete absence of silica particles in the sample since it inhibits amplification and fluorescence reading. Upon demand a Kit for DNA purification is included. Biotools recommends the use of **Speedtools Kits** product line for extraction of genomic DNA from blood (Speedtools DNA Extraction Kit: Ref. 21.131/2); from tissue (Speedtools Tissue DNA Extraction Kit: Ref. 21.136/7) from food material (Speedtools Food DNA Extraction Kit: Ref. 21.176/7) and from plant material (Speedtools Plant DNA Extraction Kit: Ref. 21.171/2).

2. FEATURES AND PRINCIPLES OF THE PROCEDURE

The QUANTIMIX LIONPROBES[®] Kit has been optimized to deliver maximum amplification efficiency, precision and sensitivity, during nucleic acid amplification in Real Time using specific probes and primers designed with LIONPROBES[®] technology

QUANTIMIX LIONPROBES[®] Kit is based on the use of a DNA polymerase (*Pfu*: aised from *Pyrococcus furiosus*) which has some enzymatic activities: 5'-3' polymerase activity, 3'-5' exonuclease activity and 3'-5' proofreading activity. During the amplification reaction the *Pfu* excises the mismatched base pairs between LIONPROBES[®] and target DNA, releasing the quencher situated at 3'-end of LIONPROBES[®]. During each amplification cycle the fluorescence emerges as result of the release of the fluorophore at 5'-end of amplification product.

3. KIT CONTENT & HANDLING INSTRUCTIONS

PLEASE CHECK INTEGRITY OF KIT AND REAGENTS BEFORE USE. DETERIORATED KITS MAY CAUSE EQUIVOCAL RESULTS.

The Kit includes the amplification reagents in liquid format. The reaction mixture is ready for use. All reagents must be stored at -20°C, thaw and handle on ice. Do not freeze/thaw repeatedly. For frequent use, we recommend aliquoting vial contents.

- **4X LIONPROBES® MIX**
Contains all necessary components for Real Time DNA Amplification assay: Biotools *Pfu* DNA Polymerase, dNTPs, MgCl₂, and LIONPROBES® Buffer. Store at -20°C.
- **50 mM MgCl₂ Solution**
Used only for specific Real Time assays, in case that an additional optimization should be required. Mix thoroughly before use. Store at -20°C.
- **5X qPCR ASTRINGENT**
Used only for specific Real Time assays, to increase the specificity in case that an additional optimization should be required. Store at -20°C.
- **qPCR ENHANCER**
Used only for specific Real Time assays, to increase the sensitivity in case that an additional optimization should be required. Store at -20°C.

REAGENTS	Ref. 10.611 (100 rxn)	Ref. 10.612 (200 rxn)	Ref. 10.613 (500 rxn)
4X LIONPROBES® MIX	550 µl	2X 550 µl	5X 550 µl
5X qPCR ASTRINGENT	550 µl	2X 550 µl	5X 550 µl
qPCR ENHANCER	30µl	2X 30µl	5X 30µl
50 mM MgCl ₂ Solution	1800 µl	2X 1800 µl	5X 1800 µl

NOTE

This product is not recommended for amplifications involving sequences homologous to Escherichia coli

4. KIT STORAGE

Upon receipt, store the different reagents under the recommended conditions (-20°C). Use non frost-free freezers. Also, for frequent use (more than once a week), aliquot the contents of the vials in different tubes, in order to avoid multiple freeze-thaw cycles.

Do not use the kit after its expiration date. If stored under the recommended conditions, the product will maintain its performance through the indicated date on the label. Do not mix reagents from other kits and/or other lots. Discard any residual amount of reagents after using the kit.

5. GENERAL GUIDELINES- Important points before starting

DNA Template and Target

- The condition of the purified DNA is a key point to obtain optimal results. Samples should be transported and stored frozen (at -20°C or at -80°C). DNA can be degraded in samples that have been stored without refrigeration. Perform the analysis shortly after the DNA extraction. In case of working with clinical samples, handle all them as if they are capable of transmitting infectious agents.
- Experimental samples may have different DNA concentrations. The quantity of template DNA to be added in each reaction depends on its purity and the experimental system used in every case. Therefore, we recommend to determine the concentration by A_{260/280} measurement or empirically.
- In case the extracted DNA can not be quantified we advice to add a fixed volume (1-10 µl), of the extraction mixture to the problem samples. The purpose of this recommendation is obtaining comparable quantitative results.
- For maximum efficiency the DNA target for Real Time PCR experiments should be 80-300 bp in length. Good results have also been obtained with longer DNA targets.

LIONPROBES® Design

- The design of the primer-probes is a very important parameter. PCR primers-probes are usually 15-30 nucleotides in length with a content of 40-60% G+C residues. To avoid primer-dimers and hairpin formation the primers should not be self-complementary or complementary to any other primer present in the reaction mixture.
- The annealing temperature of the primers should be similar (< 5°C variation), the G+C content and length has to be selected accordingly. The C and G nucleotides should be distributed uniformly.
- In most cases is recommendable and necessary to use a specialised computer program called LionSoft™ that can be downloaded from Biotools website (www.biotools.eu). This is due to the interaction between bases, salt concentration and other factors that have to be taking into account.
- The concentration range of the primers is 0.1-0.5 µM.

MgCl₂ Concentration

- 4X LIONPROBES® MIX includes MgCl₂ in its chemical composition to render a final concentration of 3 mM, which is suitable for most targets. If desired the concentration of MgCl₂ can be increased using the provided 50 mM MgCl₂ Solution.
- Optionally a standard curve of MgCl₂ concentration can be performed (use for this purpose the additional vial with MgCl₂). Recommended range: 3-6 Mm final concentration. Subtract MgCl₂ volume from the water needed to complete final reaction volume. Do not forget that a final concentration of 3 mM is obtained when using the Kit.

Real Time Amplification Program

Confirm the absence of nonspecific amplifications and primer-dimers by a melting curve analysis, using specific probes designed with LIONPROBES® technology

6. INSTRUCTION OF USE

Thaw and handle KIT components, primers and template DNA on ice. Before use mix well KIT reagents, to avoid the formation of bubbles do not use the vortex. Spin briefly KIT components in a microcentrifuge.

1.- 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. It is highly recommended to include positive and negative controls in each experiment.

Table 1. Reagents not included in the Kit (recommended concentrations)

Primers	0.1 µM – 0.5 µM final concentration
TEMPLATE DNA	depends on the experimental assay
LIONPROBES [®]	0.1 µM – 0.5 µM final concentration

Proceed to Reagent Preparation Area in a laminar flow cabinet. Use of reaction mixes and vials in non-refrigerated conditions may cause a drastic decrease in sensitivity and quality of the obtained fluorescence curves. Protect vials with LIONPROBES[®] from light at all times. Be careful not to wet the vials.

2.- For “Standard assay” aliquot **5 µl** of the **4X LIONPROBES[®] MIX** in each amplification vial. If necessary add the appropriate concentration of the reference dye.

3.- Add the primers and LIONPROBES[®] at the appropriate concentration.

Move now to the 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 amplification vials on ice until their introduction in the thermal cycler.

4.- Add to each amplification vial the **purified DNA** up to **20 µl final reaction volume** if necessary complete with sterile bidistilled water.

Table 2. Set up of the standard reaction

COMPONENT	FINAL CONCENTRATION	FINAL VOLUME REACTION (20 µl)*
4X LIONPROBES [®] MIX	1X	5 µl
PRIMER Reverse	0.1 µM – 0.5 µM	variable
LIONPROBES [®]	0.1 µM – 0.5 µM	variable
TEMPLATE DNA	variable	variable
Sterile Bidistilled Water	-	To make final rxn volume (20 µl)

*In case of larger reaction volumes, scale the indicated volumes keeping the proportion between them.

QUANTIMIX LIONPROBES[®] Kit also includes additional reagents to improve the optimization of amplification reaction:

- Add 5X qPCR ASTRINGENT to increase the specificity of the LIONPROBES[®] assay. Recommended rank: 0.25 – 1 M (final concentration)
- Add qPCR ENHANCER to increase the sensitivity of the LIONPROBES[®] assay. Recommended rank: 0.2 µl – 1 µl per reaction
- Add 50 mM MgCl₂ Solution to increase amplification signal of the LIONPROBES[®] assay. Recommended rank: 3-4 mM (final concentration)

5.- Close the amplification vials and gently mix (do not vortex) without creating bubbles. Centrifuge the amplification vials briefly if needed.

Finally proceed to Amplification Area

6.- Place amplification vials in the thermal cycler. Establish the desired thermal cycler program for amplification of the samples and start the process (see the following table).

Table 3. Real Time amplification program

Cycle	Cycle Point
Hold @94-98°C, 2-10 min 0 secs	
Cycling (30-45 repeats)	Step 1 @ 94-98°C, hold 5-15 secs, Not Acquiring
	Step 2 @ X°C*, hold 30-70 secs, acquiring reporter's fluorescence

* annealing/ extension temperature represented by an "X" may be the average of the Tm of the LIONPROBES® and the primer reverse used in the assay.

If negative controls amplify, it is possible to build a Melting curve, using a specific design of LIONPROBES®.

Table 4. Melting Programm

Cycle	Cycle Point
Dissociation (60-95°C)	Acquire from detector

7. INTERPRETATION OF RESULTS

NOTE

This protocol has been optimised for the following Real Time quantification equipments: Rotor-Gene 3000 and 6000 (Corbett Research) and ABI PRISM 7000 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. Follow therefore, the instructions and advice provided by the manufacturer.

A. Reaction Mixture Optimisation

When an optimisation of the amplification reaction is required, we suggest considering the following recommendations:

- Add 5X qPCR ASTRINGENT to increase the specificity of the LIONPROBES[®] assay. Recommended rank: 0.25 – 1 M (final concentration)
- Add qPCR ENHANCER to increase the sensitivity of the LIONPROBES[®] assay. Recommended rank: 0.2 µl – 1 µl per reaction
- Add 50 mM MgCl₂ Solution to increase amplification signal of the LIONPROBES[®] assay. Recommended rank: 3-4 mM (final concentration)
- Modify the annealing/extension temperatures and times, as well as the slopes between the amplification cycles.
- Modify the concentration of the primers and LIONPROBES[®]. Recommended rank: 0.1- 0.5 µM.

B. Additional Notes

- Experimental samples may have different DNA concentrations. The quantity of template DNA to be added in each reaction depends on its purity and the experimental system used in every case. Therefore, we recommend to determine the concentration by A_{260/280} measurement or empirically.
- In case that the extracted DNA can not be quantified we advice to add a fixed volume of the extraction mixture to the problem samples. The purpose of this recommendation is to obtain comparable quantitative results.

8. MATERIALS REQUIRED BUT NOT PROVIDED

NOTE

For all equipment, regular maintenance and calibration is necessary. Follow manufacturer's instructions, and check working parameters regularly, especially thermal cyclers and pipettes. Maintenance and calibration of instruments allows their correct functioning, and helps to detect problems that may render an incorrect analysis result.

Pre-amplification Area (DNA Purification and Reagent Preparation Areas)

- Equipment, reagents and disposable material necessary for DNA purification (depending on the method, follow manufacturer's instructions).
- Timer
- Automatic pipettes¹ (10, 20 and 200 µl), filter or positive displacement tips, RNase-free²
- Disposable examination gloves, powder-free.
- Sterile bidistilled water³ or equivalent.

¹ Precision of automatic pipettes must be in the range of 3 % of the indicated volume. If necessary, calibrate and check regularly, following manufacturer's instructions. It is recommended to use RNase-free filter tips and positive displacement tips, in order to avoid cross contamination between samples and amplicons.

² It is recommended to use different sets of pipettes for each reaction step (pre-amplification, amplification), in order to avoid contaminations that may render false positive results.

³ Available in Biotools catalog (Ref. 20.033).

- Screw cap polypropylene tubes, 1.5 ml capacity, non siliconised, conical, sterile, RNase-free. It is recommended to use screw cap tubes, in order to avoid the potential contamination of samples and controls.
- Racks for reaction vials.
- Containers for disposal of potentially-infectious material.
- Disposable filter paper for working surface, cleaning paper for accidental spills.
- Termi-DNA-Tor⁴ or equivalent, in order to remove DNA from working surfaces.

Amplification Area

- Real-time thermal cycler Rotor-Gene 3000 and 6000 (Corbett Research) or ABI PRISM 7000 series (Applied Biosystems). The use of this kit in other equipments has not been tested. For further information, contact our Technical Dpt. (info@biotools.eu).
- Laminar flow cabinet.
- Racks for reaction vials.
- Real Time amplification vials (as per manufacturer's requirements).
- Sterile bidistilled water or equivalent.
- Automatic pipettes (10, 20 and 200 µl), filter or positive displacement tips, RNase-free.
- Disposable examination gloves, powder-free.
- Containers for disposal of potentially-infectious material.
- Disposable filter paper for working surface, cleaning paper for accidental spills.
- Termi-DNA-Tor or equivalent, in order to remove DNA from working surfaces.

9. WARNINGS AND PRECAUTIONS

DNA amplification allows the amplification of minute quantities of template from a sample in an exponential manner. However, this means that foreign DNA present in the environment may also be amplified. Therefore, special laboratory practices are necessary in order to avoid false positive amplifications.

The list below contains several warnings and precautions that must be considered. For detailed information, we recommend to read the Material Safety Data Sheet (MSDS), available in our webpage (www.biotools.eu/msds.htm). Please contact our Technical Department for additional information. (info@biotools.eu).

- A. Use of dedicated micropipettes in each area (sample preparation, amplification and pre-amplification) is highly recommended.
- B. We recommend to use filter tips in order to avoid cross contamination. Pipettes must be regularly checked, in order to ensure that they are accurate within 3 % of stated volume. Use different micropipettes depending on the aliquoted volume.
- C. Negative results may occur due to the Polymerase inhibition. DNA purification must proceed in such a way that enough amount of pure DNA is obtained. It is recommended to check suitability of DNA preparations for the amplification (i.e. performance of an amplification for detection of human DNA).
- D. Follow general instructions for laboratory safety (e.g. do not eat, drink or smoke in laboratory work areas, wear disposable gloves, wear clean lab coats and eye protection, wash hands thoroughly after handling specimens and test reagents, etc.).
- E. Open and close reagent vials carefully. Follow temperature and light exposure instructions. After use, close vials and store at indicated temperatures.
- F. Do not use a kit after its expiration date.
- G. Extreme care must be taken when aliquoting the different volumes in each reaction step. Mix well after addition of each reagent, unless otherwise noted. Read instructions for use of automatic pipettes.
- H. Do not pipette by mouth.
- I. Packaging material included within the kit is resistant to the indicated storage conditions. Storage at different conditions can cause breakage of the material, and possible contamination of the kit reagents.
- J. Plastic material included within the kit is resistant under normal conditions of use. Use of plastic material in extreme conditions may cause its breakage, and therefore, the impossibility to use the kit.

⁴ Available in Biotools catalogue (Ref. 22.001/2).

- K. 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. Equipment used for amplification must remain in this area at all times.
- L. Gloves must be worn in each area and must be changed before leaving that area.
- M. As with any test procedure, good laboratory technique is essential for the proper performance of this assay. Due to the high analytical sensitivity of this test, extreme care should be taken to preserve the purity of all reagents. Discard any reagents that may be suspect for their purity.
- N. Do not touch or wet the vials in the detection areas. Use non talcum powder gloves.

10. WARRANTY

The products are warranted to the original purchaser only to conform to the quantity and contents stated on the vial and outer labels for the duration of the stated shelf life. Biotools' obligation and the purchaser's exclusive remedy under this warranty is limited either to replacement, at Biotools' expense, of any products which shall be defective in manufacture, and which shall be returned to Biotools, transportation prepaid, or at Biotools option, refund of the purchase price.

Claims for merchandise damaged in transit must be submitted to the carrier.

The product has been designed for research use only, and to be used by qualified professionals only. It is the user's responsibility to ensure that a given product is fit for a given application. Any product that does not meet the performance standards stated in the product specification sheet will be replaced at no charge. This warranty limits our liability to the replacement of the product. No other warranties of any kind, express or implied, including, without limitation, implied warranties for merchantability or fitness for a particular purpose, are provided by Biotools. Biotools shall have no liability for any direct, indirect, consequential or incidental damages arising out of the use, the results of use or the inability to use any product.

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BIOTOOLS, Biotechnological & Medical Laboratories, S.A. has been evaluated and certified to accomplish ISO 9001:2000 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

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