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HIGH SCRIPTOOLS-QUANTIMIX EASY PROBES MASTER MIX

One step quantitative RT-PCR to use with hydrolysis probes

REF.	FORMAT	CONTENT
10641	100 rxns	**High Scriptools Quantimix Easy Probes Master Mix**
10642	500 rxns	**High Scriptool Quantimix Easy Probes Master Mix**

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.

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1. DESCRIPTION

High Scriptools-Quantimix Easy Probes Master Mix (High SQEP Master Mix) is a novel real-time RT-PCR system for the quantification of RNA targets designed to be used with hydrolysis probes (e.g. TaqMan probes). The high specificity and sensitivity of this Master Mix is achieved by the use of the new Biotools High Retrotrascriptase and the Biotools HotSplit DNA Polymerase, together with a specialised buffer, in an easy-to-handle format

The Master Mix has been designed to deliver maximum efficiency, precision and sensitivity of quantitative RT-PCR. For these purpose, two high-performance enzymes, a thermostable reverse transcriptase and a DNA polymerase with hot start activity, carry out reactions. Both cDNA synthesis and PCR are performed sequentially in a single tube due to a single buffer system formulated to ensure specific primer annealing.

High Scriptools-Quantimix Easy Probes Master Mix provides a convenient and fast procedure, one step only, for synthesizing cDNA and DNA amplification in real time. All reagents necessary for both reactions are added in one tube at the same time which confers an easy-to-handle format saving manipulation time and reducing contamination risks without compromising the efficiency or sensitivity of the kit..

The Master Mix has been optimised with RNA from different origins including *RNA virus* (e.g. RNA from HCV; HIV; and H1N1 virus, among others)..

2. REAGENTS INCLUDED IN THE MASTER MIX

The system contains sufficient reagent for a number of one-tube RT-PCR reactions of 25µl and 50 µl .

- ***High SQEP Master Mix:*** An easy and convenient 2X Master Mix formulated to optimize RT-PCR reactions in a one-tube format. The buffer system and has been specifically adapted for quantitative RT-PCR analysis using sequence-specific probes. The mixture includes: Biotools HotSplit DNA Polymerase; Biotools High *Retrotranscriptase*; dNTPs; $MgSO_4$; and Reaction Buffer

- **DTT 25X Solution*:*** Provided in a separate tube

3. STORAGE AND HANDLING INSTRUCTIONS

Store all components of the *High Scriptools-Quantimix Easy Probes Master Mix* at **-20ºC**. All reagents must be thawed and handled on ice. For frequent use, divide in aliquots.

- **High SQEP Master Mix:** Mix before use.
- **DTT 25X 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: Successful reverse transcription is dependent on the integrity and purity of the template. Samples should be transported and stored frozen; if samples are stored without refrigeration, RNA can be degraded.

The Biotools HotSplit DNA Polymerase used in this system has no reverse trascriptase activity under standard reaction conditions, but amplification product will be generated out of these reactions if trace amounts of DNA with similar sequences are present in the template preparation. For optimal results, the RNA template, regardless of the type of RNA used, should be DNA-free.

RNA prepared using standard isolation techniques is a suitable substrate for amplification. Nevertheless, even trace amounts of certain agents used in RNA purification procedures (e.g. guanidine thiocyanate, phenol, EDTA, ionic detergents, silica particles) can interfere with the reaction. Biotools recommends the use of our Speedtools Total RNA Extraction kit ot Speedtools RNA Virus Extraction kit.

It is highly recommended determining the concentration by fluorimetry and to use equal amounts of RNA template. If you do not know the concentration of template RNA, add a fixed volume of the extraction mixture to problem samples. Purified RNA should be stored at -20°C or -70°C, in RNase-free water. Diluted solutions of RNA should be stored in aliquots and thawed once only. ***Relative quantification*** requires a reference sample. A reference sample provides basis for comparison in a relative quantification assay. The reference RNA should be prepared in the same manner as the experimental sample RNA. For ***absolute quantification***, use a template of known concentration as the RNA reference standard. Serial dilutions of the RNA reference standard are amplified, and results are used to generate a standard curve and determine the concentration of unknown samples. We recommend performing duplicate or triplicate amplification reactions with each dilution of the RNA standard.

The amount of template required per reaction depends upon the abundance of the RNA of interest. Up to 1µg RNA can be used in each reaction. A good starting point for a standard mass of RNA to add for an expression level of unknown abundance would be 100ng of total RNA.

$MgS0_4$ Concentration: Magnesium is required for the functioning of Biotools High Retrotranscriptase and HotSplit DNA Polymerase. A 4mM Mg^{2+} final concentration is present in the High SQEP Master Mix.

Primer and Probes Design: Prerequisites for successful RT-PCR include design of optimal primer pairs, use of appropriate primers and probes concentrations, and correct storage of primers and probes solutions

A specific primer should be used for first strand synthesis. Specific primers anneal only to defined sequences and can be used to synthesize cDNA from particular mRNAs. It is particularly important to minimise nonspecific primers and probes annealing by careful primer design. To differentiate between amplification of cDNA and amplification of contaminating genomic DNA, primers may be designed to anneal partially in two consecutive exons, avoiding contaminations with gDNA.

Regardless of primer design, the final concentration of the primer in the reaction may need to be optimised empirically; the optimal final concentration is typically between 0.4-0,8 µM (optimal concentration could vary depending on the thermal cycler used). Regarding probes concentration, the optimal concentration is typically between 0.1-0.5 µM; use 0.2 µM as starting point of optimization.

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It is recommended to use gene-specific primers designed with a Tm high enough to perform the retrotranscription at a high temperature. For the highest efficiency in real-time RT-PCR using sequence-specific probes, targets should ideally be 100-150 bp long and should not exceed 300 bp.

Synthesis of cDNA: Although High Scriptools-Quantimix Easy Probes Master Mix does not require a template denaturation step prior to initiation of the reverse transcription reaction, if desired, a denaturation step may be incorporated by incubating a separate tube containing the retrotranscription primer and RNA template at 95ºC for 2 minutes. Then, place the tube immediately on ice and add the additional reaction components.

Biotools High Retrotranscriptase synthesizes cDNA. This enzyme works in a wide temperature range, between 40-65°C, which enables to carry out the retrotranscription at a higher temperature. It minimises the effect of RNA secondary structures and encourages full-length cDNA synthesis. We recommend **45**-**47°C** for being the optimum temperature for the enzyme

The HotSplit DNA Polymerase remains completely inactive during the reverse transcription reaction and does not interfere with it. This prevents the formation of non-specific products and primer-dimers during the process. The enzyme is activated by increasing temperature during the initial denaturation step at the same time that the Biotools High Retrotranscriptase is inactivated; this ensures temporal separation of both processes and allows performance of both steps sequentially in a single tube.

Cycling Parameters: *Initial denaturation* step could be done during 5-10 min at 95°C to denature the RNA/cDNA hybrid, inactivate the Biotools High Retrotranscriptase and activate the Biotools HotSplit DNA Polymerase.

Using primers with a high Tm may be advantageous to increase *annealing and extension* temperatures. The higher temperature minimises nonspecific primer annealing and dimer formation, thus increasing the amount of specific product produced.

Most RNA samples can be detected using *45 cycles* of amplification. If only a small amount of target is available, it may be necessary to increase the number of cycles to 60. During the extension step, allow 45-60 sec for amplicons between 100-250 bp and 90 sec for amplicons > 250 bp.

5. STANDARD PROTOCOL

Materials to be supplied by user:

- Specific primers and probes
- 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. Wear disposable gloves, use nuclease-free plastic material, and filter tips.

KEEP THE REACTION VIALS REFRIGERATED until their introduction in thermal cycler. Use of reaction components in non-refrigerated conditions may cause a drastic decrease in sensitivity

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. The High SQEP Master Mix should be used at 1X. This mix works with final reaction volumes of **25** µl (see Table 1).

Proceed to the Reagent Preparation Area in a laminar flow cabinet.

1.- Thaw and thoroughly mix all reagents before dispensing.

2.- Prepare the qRT-PCR reaction mix in a sterile 1.5ml microtube on ice as indicated in Table 1. The reaction mix will be used to amplify experimental RNA, RNA reference standards, NTC and positive control reactions. Prepare sufficient reaction mix for the desired number of reactions on ice. PROTECT MIX FROM PROLONGED EXPOSURE TO LIGHT..

3.- Dispense the appropriate volume of the Reaction Mix in each reaction vial and store vials on ice. This mixture contains all components required for qRT-PCR except the template RNA.

TABLE 1. Reaction Mix preparation

COMPONENT	Final Concentration	25 µl rxn
High SQEP Master Mix	1 X	12.5 µl
DTT 25X Solution	-	1 µl
Primers	0.3-0,8 µM+	x µl
Probes	0.1-0.5 µM++	x µl
RNAse free water	-	Up to 25 µl
Template RNA	<1µg/rxn	x µl

\+ A final primer concentration of 0,5 µM is optimal for most applications.
++ A final primer concentration of 0.2 µM is optimal for most applications.

Proceed to RNA Purification Area.

Never introduce RNA in the laminar flow cabinet from the reagent preparation area. Reaction must start in the next 10 min after adding RNA and primers to the reaction mix. Keep all reagents on ice until their introduction in thermal cycler. .

4.- Add template RNA to each reaction tube. Close tubes and mix gently; centrifuge vials briefly

Proceed to Amplification Area

5.- Place tubes in thermal cycler and start the RT-PCR cycling program (See Table 2).

TABLE 2. Cycling parameters for High Scriptools-Quantimix Easy Probes Master Mix

STEP	Nº Cycles	Temperature	Time
Denaturation*	1	95ºC	2 min
Retrotranscription (synthesis of cDNA)	1	45-47ºC	30-45 min
Initial Denaturation, and inactivation of Retrotranscriptase**	1	95ºC	5-7 min
Denaturation Annealing and Extension*** (See Note 1)	35-45	94-97ºC 58-60ºC	15 -30 sec 60 sec+ FAM

* Optional: RNA and primer denaturation (see synthesis of cDNA)
** HotSplit DNA Polymerase is activated during this step
***Fluorescence Acquisition during Annealing and Extension Step (see Note 1).
\+ 45-60 sec for amplicons between 100-250 bp and 90 sec for amplicons > 250 bp.

Note 1: **When primer-dimer or nonspecific products appear, include an additional Fluorescence Acquisition Step after the Extension Step**

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

6. TROUBLESHOOTING

Little or no product detected

1. **Check template quality and quantity.** Verify the integrity of the RNA by denaturing agarose gel electrophoresis and check the quantity of your template by fluorimetry (an excess RNA can reduce RT-PCR yield).
   Carryover of reagents from some RNA purification methods can interfere with RT-PCR: reduce volume of target or change purification method.
   Ensure that reagents, tips and tubes used are RNase-free.
2. **Check primers and probes design and store conditions.** Review primers and probes design. Ensure that storage conditions are adequate.
3. **Optimise primer concentration.** Although lower primer concentration can prevent primer-dimer formation, sufficient primers are needed for successful RT-PCR. Increase primer in increments of 0.1 µM.
4. **Optimise retrotranscription conditions.** Low abundance targets, and/or templates rich in G+C content or with secondary structures often require longer retrotranscription: Increase time up to 60 min.
   If an initial denaturation/annealing step is included in the protocol, be certain to add the retrotranscriptase after the denaturation step to avoid its inactivation
5. **Optimise PCR Cycling parameters.**
   -*Increase the length of initial template denaturation up to 10 min*. Templates rich in G+C content or with secondary structures often require a longer step.
   -*Reduce the annealing temperature* in 2°C decrements.
   -*Increase N° of cycles* in increments of 5 cycles.
   -*Increase extension time* by increments of 30 sec.
   -*Check that fluorescence detection* was activated in the cycling program.
   -*Choose the appropriate filter.* Ensure that the correct channel is chosen; and that fluorescence detection takes place during the correct step.
6. **Missing reaction component.** Check reaction components, and repeat the assay.

Multiple, nonspecific amplification products

1. **Check template quality and quantity.** Verify the integrity of the RNA and check the quantity of your template Decrease the amount of RNA added to the reaction. RNA sample is contaminated with gDNA: pre-treat template with DNase.
2. **Check the design and quality of primers.** Design primers that have higher Tm and do not form hairpin loops or primer dimers.
   Check quality of primers by electrophoresis in a denaturing acrylamide gel.
3. **Increase Retrotranscription temperature.** Increase the retrotranscription temperature in 1°C increments
4. **Suboptimal reaction conditions.** Increase qPCR Astringent concentration and/or decrease the concentration of primers
5. **Optimise PCR Cycling parameters**
   - *Increase the annealing temperature* in 2°C increments.
   -*Decrease N° of cycles* in decrements of 5 cycles.
   -*Include an additional fluorescence acquisition step* (see Note 1).
6. **Decrease PCR product size.** For optimal real time PCR, design primers that amplify PCR products between 100-150 bp in length (<300 bp).

No linearity in Ct values

1. **Check template quality and quantity.** Template concentration in the reaction mix could be too low or too high.
2. **Presence of primer-dimers.** See Note 1.

Fluorescence in negative control (NTC)

1. **Contamination of some reagent.** Repeat the assay with new reagents.
2. **Presence of primer-dimers.** Include an additional data acquisition step (Note 1).

7. ORDERING INFORMATION

DESCRIPTION	Size	Reference
High SQEP Master Mix	1 x 1.4 ml	10641
	5 x 1.4 ml	10642
DTT 25X Solution	1 x 0.1 ml	10641
	5 x 0,1 ml	10642