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HIGH SCRIPTOOLS-QUANTIMIX EASY MASTER MIX

One step Quantitative RT-PCR to use with intercalating fluorophores

REF.	FORMAT	CONTENT
10611	100 rxns	**High Scriptools-Quantimix Easy Master Mix**
10612	500 rxns	**High Scriptools-Quantimix Easy 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 Master Mix (High SQE Master Mix) is a novel RT-PCR system for the detection of specific sequences within an RNA sample. This one-step, two-enzyme Master Mix uses Biotools High Retrotrascriptase and Biotools HotSplit DNA Polymerase for detection of any type of RNA sample.

The Master Mix has been designed to deliver maximum efficiency, precision and sensitivity of quantitative RT-PCR with intercalating fluorophores. 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

The real time detection of amplified products is performed by monitoring the increase of fluorescence after each amplification cycle. The fluorescent signal is generated by the incorporation of intercalating fluorophores.

The **High Scriptools-Quantimix Easy 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 a rapid and easy-to-handle format saving manipulation time and reducing contamination risks without compromising the efficiency or sensitivity of the kit.

2. REAGENTS INCLUDED IN THE MASTER MIX

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

- ***High SQE Master Mix:*** An easy and convenient 2X Master Mix formulated to setting up one-tube real-time RT-PCR. The mixture includes: Biotools HotSplit DNA Polymerase, Biotools High Retrotranscriptase, dNTPs, $MgSO_4$, and Reaction Buffer
- **DTT 25X Solution*:*** Provided in a separate tube
- **qPCR Astringent*:*** Used for specific real-time RT-PCR assays, to increase the sensitivity and especificity of RT-PCRs
- **SYBR® Green I:** intercalating fluorophore.

3. STORAGE AND HANDLING INSTRUCTIONS

Store all components of the *High Scriptools-Quantimix Easy Master Mix* a **-20ºC,** except the SYBR Green, which should be stored at 4º C.

All reagents must be thawed and handled on ice. For frequent use, divide in aliquots:

- **High SQE Master Mix:** Mix before use.
- **DTT 25X Solution:** Mix thoroughly before use.
- **qPCR Astringent:** Mix before use.
- **SYBR® Green I:** Store vial at –20ºC and avoid exposure of the vial content to light**. Add 500 µl RNase free water and mix the contents (the 1:1000 dilution is stable for 1 month at 4°C, DO NOT FREEZE).**

4. GENERAL CONSIDERATIONS

Template: Successful reverse transcription is dependent on the integrity and purity of template. Samples should be transported and stored frozen; if samples are stored without refrigeration, the RNA can be degraded.

For optimal results using this system the RNA template, regardless of the type of RNA used, should be DNA-free. The Biotools HotSplit DNA Polymerase has no reverse transcriptase 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 samples.

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 or 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. ***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 concentrations of unknown samples. We recommend performing duplicate or triplicate amplification reactions with each dilution of the RNA reference 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 correct functioning of both the Biotools High Retrotranscriptase and the HotSplit DNA Polymerase in RT-PCR reactions. A 4mM Mg^{2+} final concentration is present in the High SQE Master Mix 2X.

qPCR Astringent Concentration: The qPCR astringent can increase yield of the desired PCR product or decrease production of undesired products. Concentration of astringent has to be optimised for each reaction; use 2,5 µl for a reaction volume of 25 µl.

Primer Design: 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. To differentiate between amplification of cDNA and amplification of contaminating genomic DNA, primers may be designed to anneal partially in two consecutive exons.

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.

It is recommended to use gene-specific primer designed with a Tm high enough to perform the retrotranscription at high temperatures.

Synthesis of cDNA: High Scriptools-Quantimix Easy 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 primers 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. We recommend **45**-**47°C** apart for being the optimum temperature for the enzyme, it minimises the effect of RNA secondary structures or templates rich in G+C content and encourages full-length cDNA synthesis.

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 residual, and activate the Biotools HotSplit DNA Polymerase.

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

Most RNA samples can be detected using *40 cycles* of amplification. If only a small amount of target is available, it may be necessary to increase the number of cycles to 45-55. During the extension step, allow 45-60 sec for amplicons between 100-250 bp and 90 sec for amplicons > 250 bp.

A ***melting curve*** is essential when running assays using intercalating fluorophores in order to check the melting profile of PCR products. Take into account that addition of qPCR Astringent to reaction modifies melting temperature of specific products

5. STANDARD PROTOCOL

Materials to be supplied by user:

- *Downstream primer*
- *Upstream 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. 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 SQE Master Mix (2X) 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.-Dilute the fluorophore: Before using, dilute the stock of your intercalating fluorophore to a working solution (follow the instructions and advice provided by the manufacturer). Protect the dye from prolonged exposure to light.

3.- 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, negative and positive control reactions. Prepare sufficient reaction mix for the desired number of reactions on ice. PROTECT MIX FROM PROLONGED EXPOSURE TO LIGHT.

4.- Dispense the appropriate volume of the Reaction Mix in each reaction vial and store vials on ice.

TABLE 1. Reaction Mixture Preparation

COMPONENT	Final Concentration	25 µl rxn
High SQE Master Mix	1 X	12.5 µl
DTT 25X Solution	-	1 µl
Primers	0.4-0.8 µM	x µl
qPCR Astringent	-	2.5 µl
SYG (1/1000)[+]	-	0.8-1.3 µl
Rnase free water	-	Up to 25 µl
Template ARN	<1µg/rxn	x µl

[+] Use 1.0 µl (for V_f 25µl) for the initial optimization

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.

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

Proceed to Amplification Area

6.- Place tubes in the thermal cycler and start the cycling program (see Table 2).

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

STEP	Nº Cycles	Temperature	Time
Denaturation*	1	95ºC	2 min
Retrotranscription (synthesis of cDNA)	1	45-47ºC	30-40 min
Initial Denaturation, and inactivation of Retrotranscriptase**	1	95ºC	5-10 min
Denaturation	40-50	95ºC	10-30 sec
Annealing		2-5°C<Tm of primers	5-20 sec
Extension*** (See Note 1)		60-72ºC	45-60 sec
		84ºC	10 sec (FAM)
Melting	1	60-95ºC	Standard or programmed Melting[+]

* Optional: RNA and primer denaturation (see synthesis of cDNA)
** HotSplit DNA Polymerase is activated during this step
***Fluorescence Acquisition during Extension Step
[+] In programmed melting, start with a 0.5 ºC/sec ramp

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 design and store conditions.** Verify that the downstream primer was designed to be complementary to the downstream sequence of RNA. Design primers that have higher Tm and do not form hairpin loops or primer-dimers. 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.
   Take care with inactivation of the Biotools High Retrotranscriptase: If an initial denaturation/annealing step is included in the protocol, be certain to add the retrotranscriptase **after** the denaturation step.
5. **Suboptimal reaction conditions.**
   -Increase the concentration of the intercalating fluorophore
6. **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.
   -Choose a filter compatible with your dye. Ensure that the correct channel is activated; and that the fluorescence detection takes place during the correct step.
7. **Missing reaction component.** Check reaction components, and repeat the reaction.

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 and/or primer added to the reaction.
   If the RNA sample is contaminated with gDNA: pre-treat template with DNase I
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 100-250 bp in length (<500 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.

7. ORDERING INFORMATION

DESCRIPTION	Size	Reference
High SQE Master Mix	1 x 1.4 ml	10611
	5 x 1.4 ml	10612
DTT 25X Solution	1 x 0,1 ml	10611
	5 x 0,1 ml	10612
qPCR Astringent	1 x 270 µl	10611
	5 x 270 µl	10612
SYG	1 x 0,5 µl	10611
	2 x 0,5 µl	10612