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HIGH SCRIPTOOLS-QUANTIMIX EASY KIT

One step quantitative RT-PCR to use with intercalating fluorophores

REF.	FORMAT	CONTENT
10.621	100 rxn of 50 µl	High Scriptools-Quantimix Easy Kit
10.623	500 rxn of 50 µl	High Scriptools-Quantimix Easy Kit

Store at -20°C

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 Kit is a novel real-time RT-PCR system for the quantification of specific sequences within an RNA sample. This one-step, two-enzyme system uses High SQ Retrotranscriptase and Biotools HotSplit DNA Polymerase for quantification of any type of RNA sample.

The kit 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.

High Scriptools-Quantimix Easy Kit 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 KIT

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

- **High SQ 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; dNTPs; MgSO₄; and Reaction Buffer.
- **High SQ Retrotranscriptase:** A new RNase H minus, thermostable reverse transcriptase which exhibits high affinity for RNA and works in a higher range of temperature than most commercially retrotranscriptases. The retrotranscriptase is provided in a separate tube.
- **qPCR Astringent:** Used for specific real-time RT-PCR assays, to increase the sensitivity of RT-PCRs.
- **100 mM MgSO₄ Solution:** Used only for assays which require an additional optimisation.

The intercalating fluorophore is not provided with the kit

3. STORAGE AND HANDLING INSTRUCTIONS

Store all components of the *High Scriptools-Quantimix Easy Kit* at -20°C 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.

- **High SQ Master Mix:** Mix before use.
- **qPCR Astringent:** Mix before use.
- **MgSO₄ Solution:** Mix thoroughly before use.

If stored under 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 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 using, it should be DNA-free. The polymerase used in this system 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.

The presence of carryover of reagents (e.g. SDS, NaCl, heparin, guanidine thiocyanate) from some RNA purification methods can interfere with the reaction. We recommend the use of our Speedtools Total RNA Extraction kit or Speedtools RNA Virus Extraction kit.

It is highly recommended determining the concentration by fluorimetry. 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.

MgSO₄ Concentration: The magnesium requirement of both the High SQ Retrotranscriptase and the HotSplit DNA Polymerase in RT-PCR reactions. The MgSO₄ concentration should be optimised for each experimental target/primer combination. Although 4-12 mM magnesium sulfate concentration is suitable for most applications using High Scriptools-Quantimix Easy kit, titration of it can significantly improve the sensitivity, specificity and quality of the reverse transcription and amplification products. A 6mM MgSO₄ final concentration is present in the High SQ Master Mix. However, the kit is provided with a vial with 100 mM MgSO₄ for additional optimisation.

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 5 µl for a reaction volume of 25 µl, or 10 µl for a reaction volume of 50 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 synthesise cDNA from particular mRNAs rather than from the entire mRNA population in the sample. 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 choice, the final concentration of the primer in the reaction may need to be optimised (between 0.1-0.5µM). We recommend adding 0.2 µM of primer as a starting point of optimisation.

It is recommended to use gene-specific primer designed with a Tm high enough to perform the retrotranscription at 45-47°C.

Synthesis of cDNA: High Scriptools-Quantimix Easy Kit 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 95°C for 2 minutes. The template/primers mixture can then be added to the RT-PCR reaction mix for the standard reverse transcription incubation.

The High SQ Retrotranscriptase is a thermostable reverse transcriptase working 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 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 High SQ Retrotranscriptase; 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, thus increasing the amount of specific product produced. For primers with a low Tm, it may be necessary to decrease the annealing temperature.

Most RNA samples can be detected using 40 cycles of amplification. If the target RNA is rare or 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: Intercalating Fluorophore

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

KEEP THE REACTION VIALS REFRIGERATED until their introduction in thermal cycler. Be careful not to wet the reactions vials.

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 SQ Master Mix (2X) should be used at 1X. This mix works with final reaction volumes of **25** or **50 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 Mix preparation

COMPONENT	Final Concentration	25 µl rxn	50 µl rxn
High SQ Master Mix 2X	1 X	12.5 µl	25 µl
100 mM MgSO ₄ Solution*	4-12 mM	x µl	x µl
Primers	0.1-0.5 µM	x µl	x µl
qPCR Astringent**	-	2.5-6.0 µl	5-12 µl
SYBR® Green I (1/1000) [†]	0.1-0.5 X	0.25-1.25 µl	0.5-2.5 µl
High SQ Retrotranscriptase	-	0.25 µl	0.5 µl
RNase free water	-	Up to 25 µl	Up to 50 µl
Template RNA	<1µg/rxn	x µl	x µl

*Only necessary for concentrations of MgSO₄ >6mM

**Addition of qPCR Astringent depends on your reaction

[†]Use 0.5 µl (for V_i 25 µl) or 1.0 µl (for V_i 50 µl) for the initial optimisation

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 (experimental sample, reference standard, positive control or NTC) 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 kit

STEP	Nº Cycles	Temperature	Time
Denaturation*	1	95°C	2 min
Retrotranscription (synthesis of cDNA)	1	45-47°C	30-40 min
Initial Denaturation, and inactivation of Retrotranscriptase**	1	95°C	5-10 min
Denaturation	40-50	95°C	10-30 sec
Annealing		2-5°C<T _m of primers	5-20 sec
Extension*** (See Note 1)		60-72°C	45-60 sec [†]
Melting	1	60-95°C	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 °C/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. **Suboptimal reaction conditions.**
-Increase the concentration of the intercalating fluorophore
-Optimise the concentration of MgSO₄ by doing a curve.
5. **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 High SQ Retrotranscriptase: If an initial denaturation/annealing step is included in the protocol, be certain to add the retrotranscriptase **after** the denaturation step.
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 by denaturing agarose gel electrophoresis and check the quantity of your template by fluorimetry. 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 after the extension step
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.** The concentration of template could be too high or too low.
2. **Presence of primer-dimers.** Include an additional data acquisition step.

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 SQ Retrotranscriptase	55 µl	10.621
	5 x 55 µl	10.623
High SQ Master Mix	2.8 ml	10.621
	5 x 2.8 ml	10.623
qPCR Astringent	1.2 ml	10.621
	5 x 1.2 ml	10.623
100 mM MgSO₄ Solution	1.8 ml	10.621
	1.8 ml	10.623