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HIGH SCRIPTOOLS-QUANTIMIX EASY PROBES KIT

One step quantitative RT-PCR to use with hydrolysis probes

REF.	FORMAT	CONTENT
10.671	100 rxn of 50 µl	High Scriptools-Quantimix Easy Probes Kit
10.673	500 rxn of 50 µl	High Scriptools-Quantimix Easy Probes 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 Probes Kit is a novel real-time RT-PCR system for the quantification of RNA targets in an easy-to-handle format. The kit has been designed to use with hydrolysis probes (e.g. TaqMan probes). The high specificity and sensitivity of this kit is achieved by the use of the new High SQP Retrotranscriptase and the Biotools HotSplit DNA Polymerase, together with a specialised buffer.

The kit 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 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 an easy-to-handle format saving manipulation time and reducing contamination risks without compromising the efficiency or sensitivity of the kit.

The kit 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 KIT

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

- High SQP Master Mix:** An easy and convenient 2X Master Mix formulated to facilitate both efficient reverse transcription and specific amplification in a one-tube format. OneStep RT-PCR buffer system and has been specifically adapted for quantitative RT-PCR analysis using sequence-specific probes. The mixture includes: Biotools HotSplit DNA Polymerase; dNTPs; MgSO₄; and Reaction Buffer.
- High SQP 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 RT enzyme is provided in a separate tube.
- 100 mM MgSO₄ Solution:** Used only for assays which require an additional optimisation.

3. STORAGE AND HANDLING INSTRUCTIONS

Store all components of the *High Scriptools-Quantimix Easy Probes 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 SQP Master Mix:** Mix before use.
- MgSO₄ 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 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 the template preparation. For optimal results the RNA template, regardless of the type of RNA using, it should be DNA-free.

The presence of carryover of reagents (e.g. SDS, NaCl, heparin, guanidine thiocyanate) from some RNA purification methods can interfere with RT-PCR. 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. Purified RNA should be stored at -20°C or -70°C, in RNase-free water. Diluted solutions of RNA (e.g. dilution series used as standards) 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.

MgSO₄ Concentration: The magnesium requirement of both the High SQP Retrotranscriptase and the HotSplit DNA Polymerase. We recommend starting with an initial Mg²⁺ concentration of 6 mM as provided by the 2X High SQP Master Mix. For a few targets, reactions may be improved using Mg²⁺ concentration up to 12 mM; the kit is provided with a vial of MgSO₄ for additional optimisation.

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

A specific primer, which anneals in a defined sequence, should 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 gDNA primers may be designed to anneal partially in two consecutive exons. It is particularly important to minimise nonspecific primer and probe annealing by careful primer design.

Regardless of primer choice, the final concentration of the primer in the reaction may need to be optimised (between 0.1-0.5 µM). Depending on the real-time cyclers used, optimal primer concentrations may vary. The optimal concentration of probes is between 0.1-0.5 µM; use 0.2 µM as starting point of optimisation.

It is recommended to use gene-specific primers designed with a T_m 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: High Scriptools-Quantimix Easy Probes 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 the retrotranscription primer and RNA template at 95°C for 2 minutes. The template/primer mixture can then be added to the RT-PCR reaction mix for the standard protocol.

The High SQP 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.

The HotSplit DNA Polymerase is provided in an inactive state and has no enzymatic activity at room temperature. The enzyme 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 reaction setup, reverse transcription, and the first denaturation step. The enzyme is activated during the initial denaturation step (5-10 minutes at 95°C) at the same time that the High SQP Retrotranscriptase is inactivated, ensuring temporal separation of both process and allowing both steps to be performed 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; inactive the High SQP Retrotranscriptase; and activate the Biotools HotSplit DNA Polymerase.

Using primers with a high T_m may be advantageous to increase annealing temperature. The higher temperature minimises nonspecific primer annealing, thus increasing the amount of specific product produced. For primers with a low T_m, it may be necessary to decrease the annealing temperature.

Most RNA samples can be detected using 45 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 up 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. Be careful not to wet 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 SQP 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.-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.

TABLE 1. Reaction Mix preparation

COMPONENT	Final Concentration	25 µl rxn	50 µl rxn
2X High SQP Master Mix	1 X	12.5 µl	25 µl
100 mM MgSO ₄ Solution*	4-12 mM	x µl	x µl
Primers	0.2-1.0 µM*	x µl	x µl
Probes	0.1-0.5 µM**	x µl	x µl
High SQP 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
*A final primer concentration of 0.4 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 (experimental sample, reference standard, positive control or NTC) 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 (Table 2).

TABLE 2. Cycling parameters for High Scriptools-Quantimix Probes 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 Annealing and Extension*** (See Note 1)	40-60	95°C 60-65°C	10-30 sec 45-60 sec*

* 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 probe 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. Suboptimal reaction conditions.
-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 SQP 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.
-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.
7. 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 by denaturing agarose gel electrophoresis and check the quantity of your template by fluorimetry. 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 T_m 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. Decrease the concentration of primers and/or probe in the reaction.
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 100-150 bp in length (<300 bp).

No linearity in Ct values

1. Check template quality and quantity. The concentration of RNA 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 SQP Retrotranscriptase	55 µl	10.671
	5 x 55 µl	10.673
High SQP Master Mix	2.8 ml	10.671
	5 x 2.8 ml	10.673
100 mM MgSO ₄ Solution	1.8 ml	10.671
	1.8 ml	10.673