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HIGH SCRIPTOOLS-QUANTIMIX PROBES EASY KIT

One step quantitative RT-PCR to use with hydrolysis probes

PRODUCT	FORMAT	REF.
High Scriptools-Quantimix Easy Probes Kit	100 rxn	3B135
High Scriptools-Quantimix Easy Probes Kit	500 rxn	3B136

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 a High SQP Retrotranscriptase and 3B 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. To use it simply add to a vial containing the prepared Master Mix, the desired primers; probes; and RNA template.

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 each.

- ***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: 3B HotSplit DNA Polymerase, dNTPs, $MgSO_4$, 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 $MgSO_4$ 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 **-20ºC** 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.
- **$MgSO_4$ 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 have been 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.

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 an RNA sample 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 reference standard.

The amount of RNA 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: The magnesium requirement of both the High SQP Retrotranscriptase and the HotSplit DNA Polymerase in RT-PCR reactions. We recommend starting with an initial Mg^{2+} concentration of 6 mM as provided by the 2X High SQP Master Mix. For a few targets, reactions may be improved using Mg^{2+} concentration up to 12 mM. The kit is provided with a vial with 100 mM $MgSO_4$ for additional optimization.

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 should be used for first strand synthesis. Specific primers anneal only to defined sequences and can be used to synthesize 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, in appropriated cases 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. Designed primers or probes should be anneal to cDNA synthesised from splised mRNAs, but not to genomic DNA. Thus, detection of contaminating DNA is eliminated.

Regardless of primer choice, the final concentration of the primer in the reaction may need to be optimized (between 0.05-0.5µM). We recommend adding 0.2 µM of primer as a starting point of optimization. Depending on the real-time cycler used, optimal primer concentrations may vary.

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: 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 95ºC for 2 minutes. The template/primer mixture can then be added to the RT-PCR reaction mix for the standard reverse transcription incubation at 45-47°C. The High SQP Retrotranscriptase is a thermostable reverse transcriptase working in a wide temperature range, between 40-65°C. We recommend 45-47°C, optimal temperature for High SQP Retrotranscriptase, to minimised effects of RNA secondary structures and to encourage 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 by a 5-10 minutes, 95°C incubation step. The hot start also inactivates the reverse transcriptase enzymes, ensuring temporal separation of reverse transcription and PCR, 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 3B HotSplit DNA Polymerase.

Using primers with a high Tm may be advantageous to increase *annealing* and *extension* 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 to allow the primer to anneal to the target template.

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.

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. The Reaction Mix contains all reagents necessary for the RT-PCR except the template. ***TABLE 1. Reaction Mix preparation***

COMPONENT	Final Concentration	50 µl rxn
2X High SQP Master Mix	1 X	25 µl
100 mM $MgSO_4$ Solution*	4-12 mM	x µl
Primers	0.2-1.0 µM$^+$	x µl
Probes	0.1-0.5 µM^{++}	x µl
High SQP Retrotranscriptase	0.5 µl/reaction	0.5 µl
Nuclease free water	variable	Up to 50 µl
Template RNA	<1µg/reaction	x µl

*Only necessary for concentrations of $MgSO_4$ >6mM
$^+$A final primer concentration of 0.4 µM is optimal for most applications. However, for individual determination of best concentration, a primer titration can be performed.
$^{++}$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.

5.-Centrifuge amplification vials briefly.

Proceed to Amplification Area

6.-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	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 Annealing and Extension*** (See Note 1)	40-60	95ºC 60-65ºC	10-30 sec 45-60 sec$^+$

* Optional: RNA and primer denaturation (see synthesis of cDNA) ** HotSplit DNA Polymerase is activated during this heating 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 are RNase-free.
2. **Check primers and probes design and store conditions.** Review primers and probe design (see Primer 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. **Suboptimal reaction conditions.**
   -*Optimise the concentration of* $MgSO_4$ 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 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, primers and/or probe 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.** Decrease the $MgSO_4$ concentration 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 is 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.