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HIGH SCRIPTOOLS-QUANTIMIX EASY KIT

One step quantitative RT-PCR to use with intercalating fluorophores

PRODUCT	FORMAT	REF.
High Scriptools-Quantimix Easy Kit	100 rxn	3B131
High Scriptools-Quantimix Easy Kit	500 rxn	3B132

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 Retrotrascriptase and 3B 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 some intercalating fluorophore.

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. To use it simply add to a vial containing the prepared Master Mix, the fluorophore, desired primers, and RNA template.

2. REAGENTS INCLUDED IN THE KIT

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

- ***High SQ Master Mix:*** An easy and convenient 2X Master Mix formulated to setting up one-tube real-time RT-PCR. The mixture includes: 3B HotSplit DNA Polymerase, dNTPs, $MgSO_4$, 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 RT enzyme 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 $MgSO_4$ 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.
- **$MgSO_4$ 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. In samples that have been stored without refrigeration, 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 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.

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.

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. In general, standard curves are generated based on copy number or mass. 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 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 SQ Retrotranscriptase and the 3B HotSplit DNA Polymerase in RT-PCR reactions. The $MgSO_4$ 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 $MgSO_4$ final concentration is present in the High SQ Maste Mix. However, the kit is provided with a vial with 100 mM $MgSO_4$ 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; the recommended range of volume is 0.8-1.1 µl for RT-PCR reactions of 50µl.

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 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.

Regardless of primer choice, the final concentration of the primer in the reaction may need to be optimised (between 0.05-0.5µM). We recommend adding 0.2 µM of primer as a starting point of optimisation.

It is recommended to use gene-specific primers 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 95ºC for 2 minutes. The template/primers mixture can then be added to the RT-PCR reaction mix for the standard reverse transcription incubation at 45-47°C.

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; inactive the High SQ Retrotranscriptase; and activate the 3B HotSplit DNA Polymerase.

Using primers with a high Tm may be advantageous to increase the suggested *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 *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.

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. 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 SQ Master Mix	1 X	25 µl
100 mM $MgSO_4$ Solution*	4-12 mM	x µl
Primers	0.1-0.5 µM	x µl
qPCR Astringent**	-	0.8-1.1 µl
High SQ Retrotranscriptase	-	0.5 µl
Diluted Intercalating fluorophore^{+}	-	x µl
Nuclease free water	-	Up to 50 µl
RNA	Up to 1µg/rxn	x µl

*Only necessary for concentrations of $MgSO_4$ >6mM
**Addition of qPCR Astringer depends on your reaction
$^{+}$Final concentration: 0.1-0.5 X for SYBR® Green I or 0.5-1.5 X for Chromofy™

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 and mix gently.

6.-Centrifuge amplification vials briefly.

Proceed to Amplification Area

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

TABLE 2. Cycling parameters for High Scriptools-Quantimix 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 Extension*** (See Note 1)	40-50	95ºC 2-5°C<Tm of primers 60-72ºC	10-30 sec 5-20 sec 45-60 sec^{+}
Melting	1	60-95ºC	Standard or programmed Melting^{+}

* Optional: RNA and primer denaturation (see synthesis of cDNA)
** HotSplit DNA Polymerase is activated during this heating 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 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 $MgSO_4$ 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 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 $MgSO_4$ concentration in the reaction mix.
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 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.