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BIOTOOLS B&M Labs, S.A.
Valle de Tobalina - 52 - Nave 39
28021 Madrid
Spain

Tel. (34) 91 710 00 74
Fax (34) 91 505 31 18
E-mail: info@biotools.eu
www.biotools.eu

BIOTOOLS B&M LABS.S.A.

HIGH SCRIPTOOLS ONESTEP KIT

One Step RT-PCR

REF.	FORMAT	CONTENT
10.071	100 rxn of 50 µl	High Scriptools OneStep Kit
10.073	500 rxn of 50 µl	High Scriptools OneStep Kit

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

The kit has been designed to deliver maximum efficiency, precision and sensitivity of RT-PCR. For these purpose two high-performance enzymes, a thermostable reverse transcriptase and a DNA polymerase with hot start activity, carry out the 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 OneStep Kit provides a convenient and fast procedure, one step only, for synthesizing cDNA and DNA amplification. 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 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 50 µl. The High Scriptools OneStep Kit has been optimized for a reaction volume of 25 or 50 µl.

- ***High Master Mix:*** An easy and convenient 2X Master Mix formulated to setting up one-tube RT-PCR. The mixture includes: Biotools HotSplit DNA Polymerase, dNTPs, $MgSO_4$, and Reaction Buffer.
- ***High 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.
- ***PCR Astringent:*** Used for specific RT-PCR assays, to increase sensitivity and specificity of RT-PCRs.
- ***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 OneStep 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 Master Mix:** Mix before use.
- **PCR 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. If samples are stored without refrigeration, RNA could 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 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. 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.

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 Retrotranscriptase and the HotSplit DNA Polymerase in RT-PCR reactions. The $MgSO_4$ concentration should be optimised for each experimental target/primer combination. We recommend starting with an initial Mg^{2+} concentration of 6 mM as provided by the 2X High 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 optimisation.

PCR Astringent Concentration: The PCR 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 Vf 25 µl) or 5 µl (for Vf 50 µl) as starting point of optimization.

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, primers may be designed to anneal to sequences in exons on opposite sides of an intron.

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 primers designed with a Tm high enough to perform the retrotranscription at 45-47°C.

Synthesis of cDNA: High Scriptools OneStep 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.

The High Retrotranscriptase is a thermostable reverse transcriptase working in a wide temperature range, between 40-65°C. We recommend 45-47°C (optimum temperature) to minimised effects of RNA secondary structures and to encourage 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 Retrotranscriptase; and activate the Biotools 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.

The extension time depends on the size of the expected product; we recommend 1 min for each kb of expected product.

5. STANDARD PROTOCOL

Materials to be supplied by user:

- 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 material, and filter tips.

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

The inclusion of positive and negative controls is highly recommended in each experiment. The High 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 RT-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, NTC and positive control reactions. Prepare sufficient reaction mix for the desired number of reactions on ice.

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 Master Mix	1 X	12.5 µl	25 µl
100 mM $MgSO_4$ Solution*	4-12 mM	1-3 µl	2-6 µl
Primers	0.1-0.5 µM	x µl	x µl
PCR Astringent**	-	1-5 µl	2-10 µl
High Retrotranscriptase	-	0.25 µl	0.5 µl
Nuclease free water	-	Up to 25 µl	Up to 50 µl
RNA	Up to 1µg/rxn	x µl	x µl

*Only necessary for concentrations of $MgSO_4$ >6mM

**Addition of PCR Astringer depends on your reaction. Use 2.5 (for Vf 25 µl) or 5 µL (for Vf 50 µl) as starting point of 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.

4.-Add template RNA (experimental sample, 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 the thermal cycler and start the RT-PCR cycling program (see Table 2).

Analysis

7.-Analyse PCR products by agarose gel electrophoresis of 10% of the total reaction. Products will be readily visible by UV transillumination of an ethidium bromide-stained gel or other fluorescent intercalating.

8- Store reaction products at -20°C until needed.

TABLE 2. Cycling parameters for High Scriptools OneStep kit

STEP	Nº Cycles	Temperature	Time
Denaturation*	1	95ºC	2 min
Retrotranscription (synthesis of cDNA)	1	45-47ºC	30-60 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		60-72ºC	45-60 sec
Final Extension	1	72ºC	2-5 min
Cooling	∞	15ºC	∞

* Optional: RNA and primer denaturation (see synthesis of cDNA)

** HotSplit DNA Polymerase is activated during this step

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.**
   -Optimise the concentration of $MgSO_4$ by doing a curve.
   -Decrease PCR Astringent concentration
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 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.
7. **Thermal cycler programmed incorrectly.** Verify that times and temperature are correct.
8. **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 genomic DNA is detected, first treat the template RNA with DNase I, or if this is not possible, redesign primers to avoid amplification of gDNA.
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 PCR Astringent concentration*
   -*Decrease primer concentration*
   -*Optimise $MgSO_4$ concentration*
5. **Optimise PCR cycling parameters.**
   -Increase the annealing temperature in 2°C increments.
   -Decrease N° of cycles in decrements of 5 cycles.
6. **Contamination of some reagent.** If you view band in negative controls, repeat the assay with new reagents.

7. ORDERING INFORMATION

DESCRIPTION	Size	Reference
High Retrotranscriptase	55 µl	10.071
	5 x 55 µl	10.073
High Master Mix	2.8 ml	10.071
	5 x 2.8 ml	10.073
PCR Astringent	1.2 ml	10.071
	5 x 1.2 ml	10.073
100 mM $MgSO_4$ Solution	1.8 ml	10.071
	1.8 ml	10.073