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BIOTOOLS B&M LABS.S.A.

BIOTOOLS HIGH RETROTRANSCRIPTASE-STARTER KIT

REF.	FORMAT	CONTENT
10.081	50 rxns	**Biotools High Retrotranscriptase-Starter Kit**
10.082	100 rxns	**Biotools High Retrotranscriptase-Starter Kit**
10.091	50 rxns	**Biotools High Retrotranscriptase-Starter Kit with Random Primers**
10.092	100 rxns	**Biotools High Retrotranscriptase-Starter Kit with Random Primers**
10.061	50 rxns	**Biotools High Retrotranscriptase-Starter Kit with Oligo (dT) Primer**
10.062	100 rxns	**Biotools High Retrotranscriptase-Starter Kit with Oligo (dT) Primer**

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

BiotoolsHigh Retrotranscriptase-Starter Kit is a complete system for efficient synthesis of first strand cDNA. The kit uses the Biotools High Retrotranscriptase, a new recombinant reverse transcriptase that features a high thermostability and lacks the RNase H activity. The enzyme provides first strand cDNA synthesis with a broader optimal reaction temperature from 40°C to 65°C. Due to its thermostability, Biotools High Retrotranscriptase can transcribe GC-rich templates with large amounts of secondary structure without the help of reaction additives.

Biotools High Retrotranscriptase-Starter kit is supplied with Oligo $(dT)_{15}$ Primer and/or Random Primers. Random hexamer primers bind non-specifically and are used to synthesise cDNA from all RNAs in total RNA population. TheOligo $(dT)_{15}$ Primer selectively anneals to the 3'-end of poly(A) RNA, synthesising cDNA only from poly(A) tailed mRNA. Gene-specific primers may also be used with the kit to prime synthesis from a specified sequence. The kit contains a recombinant RNase Inhibitor, *High RNase Inhibitor*, which protects RNA from degradation by RNases A, B and C.

The first strand cDNA products generated by BiotoolsHigh Retrotranscriptase are suitable for downstream gene cloning, analysis by standard PCR or real-time PCR.

2. KIT COMPONENTS

- ***BiotoolsHigh Retrotranscriptase:***Provided in storage buffer: 5mM Tris-Acetate (pH 8.0),15mMKCl, 1.5mM EDTA, protease inhibitors, and 50% (v/v) glycerol.
- ***10X High RT Reaction Buffer:*** 100mM Tris-HCl (pH 9.0), 500 mMKCl, and 1 mM DTT.
- ***100 mMMgSO$_4$Solution***
- ***High RNase Inhibitor:*** This inhibitor is supplied at a concentration of 40 U/µl in storage buffer: 20mM HEPES-NaOH (pH 7.5), 50 mMNaCl, 8 mM DTT, 0.03% (v/v) ELUGENT™ Detergent and 50% (v/v) glycerol.
- ***Oligo $(dT)_{15}$ Primer:*** Provided with some variants of the kit in lyophilized form(30µg, 6.7nmol).
- ***Random Primers:*** Provided with some variants of the kit in lyophilized form (30µg, 17nmol).
- ***dNTP Mix, 10 mM each:*** The premixing of the four dNTPs (dATP, dCTP, dGTP and dTTP) at equal concentrations, 10 mM each,is provided in aqueous solution.
- ***Nuclease Free Water***

3. STORAGEAND HANDLING INSTRUCTIONS

Random and Oligo $(dT)_{15}$ Primers are shipped lyophilized and can be stored at 4°C or at -20°C. Reconstitute primers with nuclease-free water at a convenient concentration (e.g.0.5 µg/µl) and once they are resuspended store at -20°C.

Store all other components at -20ºC in a constant temperature freezer. All reagents must be thawed and handled on ice.

If stored under recommended conditions, the product will maintain performance through the indicated date on the label.

Avoid exposure of reagents to frequent temperature changes.For frequent use, divide in aliquots to avoid multiple freeze-thaw cycles.

4. GENERAL CONSIDERATIONS

Template:Successful reverse transcription is dependent on the integrity and purity of the template.RNA should have a minimum $A_{260}/_{280}$ ratio of 1.7 or higher. For optimal results the template, regardless of the type of RNA used, it should be DNA-free. Small amounts of genomic DNA in the RNA preparation may be amplified along with the target cDNA. To remove residual DNA treat your RNA preparation samples with RNase-free DNase.

The presence of carryover of reagents (e.g. SDS, NaCl, heparin, guanidine thiocyanate) from RNA purification methods can interfere with subsequent reactions like RT-PCR or qRT-PCR. You can remove inhibitors by precipitating the RNA before first strand synthesis including a final 70% ethanol wash step. We recommend the use of our Speedtools Total RNA Extraction kit for purification of samples.

Either total RNA or mRNA can be used in the reverse transcription reaction. Total RNA is generally sufficient for most RT-PCR analyses. The amount of RNA required per reaction depends upon the abundance of the transcript of interest; the type of sample; and the primer used by the cDNA synthesis. In general 10 ng to 5µg total RNA or 1 ng-500 ng of mRNAis recommended.

MgS0$_4$Concentration: The $MgSO_4$ concentration should be optimised for each experimental target/primer combination. We recommend starting with an initial Mg^{2+} concentration of 3mM.

Choice of Primer for Reverse Transcription: Selection of an appropriate primer for reverse transcription depends on target RNA characteristics (*e.g.*presence of secondary structure or a high percentage of GC). For in vitro applications, the primer can be either: Oligo(dT) Primer, Random Primer Mix, or a Sequence-specific Primer.

Oligo $(dT)_{15}$Primer, is used to hybridise to 3' poly(A) tails present in most eukaryotic mRNAs. Since oligo (dT) primer generally guarantees the synthesis of the longest cDNA terminating at the 3'-end of the transcript, it is the primer of choice for full-length cDNA synthesis. Recommend concentration:1-10 µM.

Random Primer Mix prime reverse transcription at multiple points along the transcript and they are useful for either long mRNA or transcripts with significant secondary structure. It offers good performance in a wide range of RNA templates. It yields shorter cDNAs on average and can be used for the detection of multiple short RT-PCR products. If you use *random primers* to cDNA synthesis, the ratio of primers to RNA may be selected to control the average length of cDNA products; higher ratios will produce smaller cDNAs. We recommend adding 150-250 ng of primers as a starting point of optimisation.

Gene Specific Primer anneals only to defined sequences and can be used to synthesize cDNA from particular mRNAs rather than from the entire mRNA population in the sample. This priming method gives good results when the amount of RNA is limiting and only one particular cDNA is desired. For specific primer the final concentration in the reaction may need to be optimisedbetween 0.1-1µM.It is recommended to use gene-specific primers designed with a Tm high enough to perform the retrotranscription at 45-47°C.

Biotools has different variants of the *Biotools High Retrotranscriptase-Starter Kit.* The most complete (*Biotools High Retrotranscriptase-Starter Kit*) is supplied with lyophilisedOligo $(dT)_{15}$ Primer and Random Primers; and the other variants include only one of these primers: Oligo $(dT)_{15}$ Primer or Random Primers (see Ordering Information-point 7).

High RNase Inhibitor: It is a recombinant mammalian ribonuclease inhibitor provided at a concentration of 40U/µl. *High RNase Inhibitor* protects RNA from degradation and improves total cDNA yields including percent total full length of cDNA. It inhibits the activity of a wide spectrum of RNases, including RNase A, RNaseB, and RNaseC by binding them in a non-competitive mode.

Common denaturants and oxidizing reagents strongly inhibit the High RNase Inhibitor and release the RNase bound. This inhibitor is also inactivated by heating at 75°C for 10 min.

Although the use of *High RNase Inhibitor* in cDNA synthesis is optional, it is strongly recommended. Working concentration: 1U/µl.

Synthesis of cDNA: Although Biotools High Retrotranscriptasedoes not require a template denaturation step prior to initiation of the reverse transcription reaction, it could be useful to ensure denaturation of RNA secondary structures. If desired, a denaturation step may be incorporated by incubating a separate tube containing primer and RNA template at 95ºC for 2 minutes orat 65ºC for 10min.Do not incubate Biotools High Retrotranscriptase enzyme at 95ºC; it will be inactivated. Then the template/primers mixture should be cooled and added to the reaction mix for the standard reverse transcription incubation.

*Temperature:*BiotoolsHigh 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 actual reaction temperature depends on the length of cDNA to be synthesized; the GC content of the target RNA; and selected primer for the reverse transcription reaction. For transcripts >4 kb, incubate the reaction at 47°C for 1h. Prolonged incubation at lower temperatures will increase the yield of full-length product.

When using *Random Primers* for first-strand cDNA synthesis, include a primer incubation step (25°C for 10 min), to maximise primer-RNA template binding, followed by 30 min at 47°C. For *Oligo(dT)$_{15}$Primer* the extension temperature may be optimised around 47°C.

Amount *of Biotools High Retrotranscriptase:*Use 0.25-1µlBiotools High Retrotranscriptase per reaction of 20 µl, depending on the template amount. Use approximately 0.5 µl for 1µgtotal RNA template.

5. PROTOCOL FOR cDNA SYNTHESIS

Materials to be supplied by user:

- Template RNA

To prevent contamination both the laboratory environment and all prepared solutions must be free of RNases: autoclave all containers and pipette tips, wear disposable gloves, use nuclease-free material and filter tips.

1.-Thaw all necessary components and place them on ice. Keep all reagents on ice after thawing and briefly centrifuge them before dispensing. A control reaction without reverse transcriptase is recommended to examine the DNA contamination in samples.

2.-**Optional:** Prepare the *template/primer mixture*in a thin-walled nuclease-free reaction tube on ice as indicated in Table 1.

TABLE 1.Preparation of template/primer mixture

COMPONENT	Final Concentration	15 µl rxn
Template RNA	variable*	x µl
Oligo (dT)$_{15}$ primer	1-10 µM	x µl
Or Random primer	150-250 ng	x µl
Or Specific primer	0.1-1 µM	x µl
Nuclease free water	-	Up to 15 µl

*****10ng-5µg total RNA or 1-500ng mRNA

3.-**Denaturation:** Incubate at 95ºC for 2 min(or at 65ºCfor 10 min), then place the tube immediately on ice.

4.-Prepare *reverse transcription reaction mix*in a thin-walled nuclease-free reaction tube on ice as indicated in Table 2. The reaction mix will be used to amplify experimental RNA, negative and positive control reactions. Prepare sufficient reaction mix for the desired number of reactions on ice.

TABLE 2.Preparation of reverse transcription reaction mix

COMPONENT	Final Concentration	20 µl rxn
10X High RT Reaction Buffer	1 X	2 µl
100mM $MgSO_4$ Solution*	3 mM	0.6 µl
dNTP Mix, 10 mM**	0.2-0.5 mM each	0.4-1 µl
High RNase Inhibitor (40U/µl)$^+$	1 U/µl	0.5 µl
Nuclease free water	-	Up to 5 µl
Biotools High Retrotranscriptase	-	0.5 µl

*****Use3mM as a starting point (up to 6 mM)
******Use 0.5mM each as a starting point
$^+$Optional

5.-Dispense 5 µl of reverse transcription reaction mix (Table 2) to each reaction tube on ice. Be careful to prevent cross-contamination.

6.-Add 15µl of RNA/primer mix (Table 1) to each reaction for a final reaction volume of 20 µl per tube.

7.-Mix well and spin the tube firefly in a microfuge.

8.-**Extension**: Incubate the 20µl cDNA synthesis reaction at 47°C for 30 min. If Random Primer is used, an incubation step at 25°C for 10 min is recommended before the 47°C incubation (see synthesis of cDNA).

9.-**Inactivation:**Inactivate Biotools High Retrotranscriptase by heating the reaction mix to 85°C for 5 min

10.-Place tubes on ice. The cDNA product should be stored at 4°C for 1-2h or at -20°C for longer periods.

11.-For downstream PCR amplification, the volume of cDNA product should not exceed 1/10 of the PCR reaction volume. However, the amount of reverse transcription reaction used in the PCR may be modified after experimental optimisation.

6. TROUBLESHOOTING

Low yield of cDNA product

1. **Check template quantity and quality.** Verify the integrity of the RNA by denaturing agarose gel electrophoresis and check the quantity of your template by fluorimetry. Repurify RNA template if the nucleic acid appears degraded.

   -An excess RNA can reduce the yield of reaction.

   -Target RNA was not present in the sample or was present at low levels. Use poli(A)+ RNA rather than total RNA as a template to increase mRNA target abundance.

   -Inhibitor was present in sample: Reduce template volume in the reaction; perform an additional purification step (ethanol precipitation); or change purification method. Ensure that reagents, tips and tubes are RNase-free.

2. **RNasecontamination.** Protect RNA from ribonuclease degradation during the cDNA reaction by adding *High RNase inhibitor*. An excess of some RNaseinhibitors can interfere with RT-PCR reactions

   Use RNase-free plastic material.

3. **Problems with the reverse transcription primer:** *Check primers design; store conditions; and concentration.*

   -Verify that the gene-specific primer is able to bind to the mRNA (complementary to the downstream RNA sequence).

   -Try another gene-specific primer or switch to an anchored-Oligo (dT)$_{15}$Primer or random hexamers.

   -Primer concentration was too low. Increase primer concentration in the reaction.

   -Poor primer annealing. If Oligo(dT)$_{15}$Primer or Random Primer were used, verify that times and temperatures of incubation are correct.

   -Ensure that storage conditions are adequate.

4. **Suboptimal reaction conditions.**
   -Optimise Mg^{2+} concentration, annealing temperature and extension time.

   -Low abundance targets, *and/or templates rich in GC content or with secondary structures* often require longer retrotranscription: Increase time up to 60 min.Prolonged incubation at lower temperatures will increase the yield of full-length product (≤60 min).

   -If an *initial denaturation/annealing step*is included in the protocol, be certain to add Biotools High Retrotranscriptaseand High RNase Inhibitor **after** the denaturation step.

5. **Optimise enzyme concentration.** Do not use more than 0.5µl Biotools High Retrotranscriptase to transcribe 1 µg total RNA template in a 20 µl cDNA synthesis reaction. For a different amount of template, modify the amount of retrotranscriptase in the reaction.

6. **Missing reaction component.** Always perform a positive control reaction with a template/primer combination that has amplified well in previous assays to determine when a component was omitted. Check reaction components, and repeat the reaction.

7. **Thermal cycler programmed incorrectly.** Verify that times and temperatures are correct.

7. ORDERING INFORMATION

Components	References					
	10.081	**10.082**	**10.091**	**10.092**	**10.061**	**10.062**
High Retrotranscriptase	30 µl	55 µl	30 µl	55 µl	30 µl	55 µl
10 X High RT Reaction Buffer	1.8 ml	1.8 ml	1.8 ml	1.8 ml	1.8 ml	1.8 ml
100 mM $MgSO_4$ Solution	1.8 ml	1.8 ml	1.8 ml	1.8 ml	1.8 ml	1.8 ml
High Rnase Inhibitor (40U/µl)	1 x 1000U	2 x 1000U	1 x 1000U	2 x 1000U	1 x 1000U	2 x 1000U
dNTP Mix, 10 mM each	1 x 60 µl	2 x 60 µl	1 x 60 µl	2 x 60 µl	1 x 60 µl	2 x 60 µl
Nuclease Free Water	2 x 1.8 ml	4 x 1.8 ml	1 x 1.8 ml	2 x 1.8 ml	1 x 1.8 ml	2 x 1.8 ml
Oligo (dT)$_{15}$ Primer	1 x 30 µg	2 x 30 µg			1 x 30 µg	2 x 30 µg
Random Primers	1 x 30 µg	2 x 30 µg	1 x 30 µg	2 x 30 µg		