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3B High Retrotranscriptase

PRODUCT	FORMAT	REF.
3B High Retrotranscriptase	100 rxn	3B120
3B High Retrotranscriptase	500 rxn	3B121

1. DESCRIPTION

3B BlackBio Biotech High Retrotranscriptase is a thermostable, new recombinant reverse transcriptase expressed in E. coli.

The enzyme is an RNase H minus which exhibits high affinity for RNA and works in a higher range of temperature than most commercially retrotranscriptases. Due to its thermostability, 3B High Retrotranscriptase can transcribe GC-rich templates with large amounts of secondary structure without the help of reaction additives.

3B High Retrotranscriptase will accept three different types of primer for cDNA synthesis: Oligo (dT) primer; random hexamer primers; or a gene-specific primer. The enzyme can be used at a temperature up to 65°C, and it is recommended for RT-PCR or qRT-PCR.

Product applications:

- Synthesis of first strand cDNA for use in subsequent amplification reactions: RT-PCR or qRT-PCR.
- RT-PCR amplification of difficult RNA templates, such as GC-rich templates with large amounts of secondary structure
- Incorporation of Cy3-, Cy5-, Biotin-, or aminoallyl-modified nucleotides during cDNA synthesis (e.g., for use in microarray hybridization).

2. REAGENTS INCLUDED IN THE KIT

- **3B BlackBio Biotech High Retrotranscriptase:** Provided in storage buffer. Storage buffer: 5 mM Tris-Acetate (pH 8.0), 15 mM KCl, 1.5 mM EDTA, protease inhibitors, and 50% glycerol (v/v).
- **10X High RT Reaction Buffer:** 100 mM Tris-HCl (pH 9.0), 500 mM KCl, and 1 mM DTT.
- **100 mM $MgSO_4$ Solution**

3.STORAGE CONDITIONS

Store the product at -20ºC in a constant temperature freezer until the expiration date printed on the label.
Avoid exposure to frequent temperature changes.

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 that have been stored without refrigeration, RNA can be degraded. For optimal results using this system, the template, regardless of the type of RNA used, 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 some 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.

It is highly recommended determining the RNA concentration by fluorimetry. If you do not know the concentration of template, 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; the type of sample (total RNA or mRNA); and the primer used by the cDNA synthesis. A good starting point for a standard mass of RNA to add for an expression level of unknown abundance would be 100 ng of total RNA (10 ng-5 ug) or from 1 ng-100 ng of mRNA.

$MgS0_4$ Concentration: The $MgSO_4$ concentration should be optimised for each experimental target/primer combination. We recommend starting with an initial Mg^{2+} concentration of 3 mM. For a few targets, reactions may be improved using Mg^{2+} concentration up to 6 mM; the enzyme is provided with a vial of 100 mM $MgSO_4$.

Primer Design: 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), which hybridizes to the poly(A)+ tails of eukaryotic mRNAs, random hexamers, which prime synthesis throughout the length of the RNA template, or a sequence-specific primer, which hybridizes to a known sequence within the RNA template.

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. For specific primer the final concentration in the reaction may need to be optimised, between 0.05-0.3µM; we suggested 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.

For *oligo* $(dT)_{15}$ *primer* we recommend a concentration of 1-10 µM. *Random hexamers prime* reverse transcription at multiple points along the transcript and they are useful for either long mRNA or transcripts with significant secondary structure. 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. If you want to synthesise longer cDNAs, you can decrease the concentration of random primers down to minimum of 0.1 µg in the cDNA synthesis reaction. We recommend adding 3.0 µg of primers as a starting point of optimisation.

Synthesis of cDNA: Although **3B High Retrotranscriptase** does 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 or at 65ºC for 10min. Do not incubate 3B High Retrotranscriptase enzyme at 95ºC; it will be inactivated. Then the template/primers mixture can be cooled to reach the selected temperature for retrotranscription and added to the reaction mix for the standard reverse transcription incubation.

Temperature: *3B High 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 hexamer primers* reduce the incubation temperature to allow efficient annealing, we recommend a two-step incubation: 10 min at 25°C, followed by 30 min at 47°C. For oligo$(dT)_{15}$ primer the extension temperature may be optimised between 37-47°C.

Amount of 3B High Retrotranscriptase: Use 0.25-1µl of our Retrotranscriptase per reaction of 20 µl, depending on the template amount. Use approximately 0.5 µl for 1µg total RNA template.

5. PROTOCOL FOR cDNA SYNTHESIS

Materials to be supplied by user:

- Primer
- dNTP Mix
- Template RNA
- Ribonuclease inhibitor (optional)
- Nuclease-free water

To minimize the risk of RNase contamination autoclave all containers and pipette tips that will used in the cDNA synthesis reaction. Wear disposable gloves and 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.

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	20 µl rxn
Template RNA	variable*	x µl
Oligo $(dT)_{15}$ primer	1-10 µM	x µl
Or Random primer	3 µg	x µl
Or Specific primer	0.05-0.3 µM	x µl
Nuclease free water		Up to 15 µl

* Use 3mM as a starting point

**We recommend 0.2-1mM of each; use 0.2-0.5mM each as a starting point.

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

6.-Add 15 µl of RNA and primer mix 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 for 30 min at 47°C. For random hexamers incubate 10 min at 25°C followed by 30 min at 47°C (see synthesis of cDNA)

9.-**Inactivation:** Inactivate of 3B High Retrotranscriptase by heating the reaction mix to 85°C for 5 min

10.-Place the tube on ice. At this point the reaction tube may be stored at 4°C for 1-2h or at -20°C for longer time periods.

11.-Use **1-5 µl** of cDNA reaction in the subsequent PCR or qPCR. 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 RT yield; if you must use lower amounts of RNA, you may obtain better results by priming with a gene-specific primer.
   -*Target RNA was not present in the sample or was present at low levels.* Use poly(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. **RNase contamination.** Protect RNA from ribonuclease degradation during the cDNA reaction by adding some protector RNase inhibitor. An excess of some inhibitors 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) primer or random hexamer primers.
   -*Primer concentration was too low.* Increase primer concentration in the reaction.
   -*Poor primer annealing.* If oligo(dT) primer or random hexamers were used, verify that times and temperatures 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 G+C 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 (≤62 min).
   -If an *initial denaturation/annealing step* is included in the protocol, be certain to add the 3B BlackBio Biotech High Retrotranscriptase **after** the denaturation step.
5. **Optimise enzyme concentration.** Do not use more than 0.5µl 3B High Retrotranscriptase to transcribe 1 µg total RNA template in a 20 µl cDNA synthesis reaction. For a different amount of template, modify proportionally 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.